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Annual Report of the Scientific Director for General Laboratories and Clinics, National Cancer Institute July 1, 1967 through June 30, 1968

This annual report of research activities within the General Laboratories and Clinics program area of the National Cancer Institute for the most part consists of descriptive analyses of laboratory and clinical investigations carried out during the past year. It does not review the searching analysis by members of the scientific and administrative staff in regard to the task of planning a continuing dynamic program of cancer research.

Planning intramural research programs with a particular thrust against cancer in the past has been accomplished within the process of program growth itself, where expanding facilities and resources allowed changes in research direction through simple addition of new activities. Current rates of expansion, now at a plateau, no longer allow changes by program additions without other alterations, that is, by restructuring existing program elements to one degree or another. Therefore, a great deal of attention is required on a continuing basis to insure maximum utilization of relatively fixed resources. Encouragement of new kinds of research may at times require discouragement of other research. Recruitment of research staff requires greater attention to the kinds of talent sought, in addition to the usual insistence on quality of performance. And perhaps most important is the need to find organizational structures sufficiently strong, but adequately flexible, to permit ease of adjustment into changing areas of research undergoing changing degrees of emphasis.

Members of the staff of the General Laboratories and Clinics have given serious thought to these problems, recognizing the need to cope with both the science of laboratory and clinical research, and the science of planning for that research.

SUMMARY
Annual Report July 1, 1967 through June 30, 1968
Macromolecular Biology Section, GL&C, NCI

Research in the Macromolecular Biology Section continued on selected aspects of molecular changes taking place during both neoplastic cell transformation and also during embryonic differentiation. There were two aspects of cell biochemistry which were emphasized. One pertained to the earliest detectable differences on the nucleic acid level, and the second to changes on the cell surface.

The approach on the nucleic acid level is illustrated by the work of Drs. Smith and Mora. Rat liver chromatin was prepared in the most "natural" form feasible, and then it was studied to determine what portion of the DNA is available for RNA transcription *in vitro*. This was found to represent only about 5% of the DNA. Comparing this with earlier work by others, it is almost certain that the same 5% is used which is transcribed *in vivo*. Then the differences were studied which develop in transcription during neoplastic transformation. Normal rat liver chromatin was used *in vitro*, to synthesize highly radioactive RNA, and this labelled RNA was then hybridized to liver DNA in the presence of other unlabelled RNA molecules as competitors. The competitor RNA preparations were from both the normal rat liver and also from a hepatoma, and from each of these two tissues from both the cytoplasmic and from the nuclear fraction. There were revealing differences in the various RNA competition curves. This confirmed the expectation that, similar to tissue differentiation, characteristic differences develop in the transcription of the DNA during the neoplastic transformation. Further defining of these differences is our next goal.

The possible role of mitochondrial DNA in differentiation and neoplastic transformation was investigated by Drs. Leffler and Mora. Great emphasis was placed upon producing pure mitochondrial DNA, free from traces of nuclear DNA, so that the specific message formed on this mitochondrial DNA can be followed and studied in the cell. We achieved excellent purification of the DNA of the mitochondria from rat liver and kidney. This DNA is small, about 10 million in molecular weight, and occurs in the native form as a double stranded "super-twisted" DNA with both strands closed in a continuous circular form. After one nick was produced in one of the DNA strands, we could separate the two strands of rat liver mitochondrial DNA by alkaline cesium chloride density gradient centrifugation. This now permits, for the first time on a DNA isolated from a higher organism, the determination of which one of the two DNA strands is the "sense" strand, that is: which one is being transcribed to RNA. We plan

next to follow this mitochondrial RNA message in differentiated cells such as liver, kidney, etc., and to clarify the role of this RNA in the particular cells. Current reports from other laboratories imply that RNA homologous to mitochondrial DNA may have a role in the synthesis of cytoplasmic and also of intracellular membranes. In other work on mitochondrial DNA, our study by nucleic acid hybridization techniques virtually assured a conclusion that the polyoma genome is not inserted into the mitochondrial DNA during neoplastic cell transformation by polyoma DNA, but apparently it is carried within the nucleus of the neoplastic cell.

The work of Dr. Luborsky on the RNA of tumor viruses continued with two objectives in mind. One, to characterize the intact high molecular weight RNA by accurate physico-chemical techniques so as to allow detection of possible differences between the various avian and murine sarcoma and leukemia virus RNA molecules. Second, to see whether such intact RNA molecules could be made to penetrate cell surfaces to infect a cell in tissue culture, so that the molecular biology of the RNA reproduction, including also the cell transformation mechanism, could be studied under controlled conditions. A combination of refined ultracentrifugal characterization techniques and studies of cell culture conditions which enhance cell membrane permeability were used by Dr. Luborsky in working on these problems. His studies help to provide the necessary foundation for the study of the molecular biology of these large RNA containing animal tumor viruses, following the pioneering work on the smaller and simpler RNA bacteriophages done by Spiegelman and others.

The messenger RNA in the cell interacts with the ribosomes, and with the help of the transfer RNA (tRNA) specifies the synthesis of the various cell proteins. Dr. Pestka continued his careful and elegant studies in this field which he started in Dr. Nirenberg's laboratory. These deal with the numerous factors which influence macromolecular interactions in the *in vitro* protein biosynthetic system isolated from *E. coli*. A new application of this work was begun, carried out mainly in the Medicine Branch. This compares normal and leukemic leukocytes to see if there are any new tRNA species or any differences in the amino acid synthetases, the specific enzymes which attach the amino acids to the tRNA.

To some extent, control of the translation of RNA into proteins, in bacterial systems, is known to depend on the allosteric transition of certain enzymes. One such transition is exhibited by the subunits of β-galactosidase from *E. coli*. Dr. Shifrin finished a fruitful line of work in this field during which he observed extremely high molecular weight aggregates of the β-galactosidase subunits. Also, unexpectedly, he found that under certain conditions there is a new enzyme activity, a

transferase activity exhibited by β-galactosidase.

Dr. Shifrin's experience in the study of interaction of proteins is going to be useful in a new field he has decided to enter: the study of cell surfaces in differentiation. During the last few months Dr. Shifrin has been collaborating with investigators at the Carnegie Institution of Washington, Department of Embryology, Baltimore, Md. He is exploring experimental systems in which he can begin to study the earliest changes in the cell surface in higher organisms after the fertilization of the egg cell. Changes in mutual adhesion of the cells and modification of chemical constituents in the cell surface must occur, which are responsible for the movement and organization of the cells in embryogenesis. Obviously, very likely the same types of chemical methods can be used to detect changes in the neoplastic transformation of cells, for example, in determining why cells migrate into new sites and multiply in metastases, or in determining why cells lose inhibition of their growth by the neighboring cells.

In this last context we already have another line of work going on in our laboratory on a suitably selected experimental system. We have studied cell surface changes when certain mouse fibroblast cells were grown in tissue culture, before and after transformation with polyoma virus. First, we have determined that no macromolecular substance is being released from the normal cells, which stops their growth in dense cultures. Apparently actual intimate cell membrane contact is necessary between these cells to stop growth. Most of the metabolic processes have to be maintained in the cells for this "contact" inhibition, although DNA synthesis is not essential. However, replenishment of some apparently unstable surface component is essential, which then remains as part of the cell surface. In comparing the normal fibroblasts and the cells transformed with polyoma or SV 40 viruses, interesting differences were detected in the glycolipid components and in the acid mucopolysaccharides, both characteristic components of cell membranes. Collaborative undertakings are in progress to define the chemical nature of these changes.

Characterization of cell membranes and cell surface protein components, mainly by electronmicroscopy, is being carried out by Dr. Marchesi and his collaborators. He has succeeded in isolating a novel fibrous protein present in the red cell membrane. He is expanding these studies to isolate cytoplasmic cell membranes and to fractionate a similar cell membrane protein from other cultivated cells. He also has become interested in the acid mucopolysaccharide complexes. While Dr. Marchesi is going to be transferred soon to NIAMD to head a Section in the Laboratory of Experimental Pathology, we plan to continue collaboration. We are also recruiting an electronmicroscopist for his replacement.

Publications:

Mora, P. T.: Nucleic Acids and the Biosynthesis of Proteins. (A General Review) In Laki, K. (Ed.): Fibrinogen. New York, N. Y., Marcel Dekker, Inc., 1968, pp. 241-267.

Serial No. NCI-3101-D
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Nucleic Acid Homologies Between Normal Rat Liver and Morris Hepatoma 5123.

Previous Serial Number: Same

Principal Investigator: Carl E. Smith

Other Investigators: Dr. Peter T. Mora; Dr. Stanley Shackney, Cancer Therapy Evaluation Branch, NCI, NIH.

Man Years (July 1, 1967 through June 30, 1968)

Total:	2
Professional:	1 1/4
Other:	3/4

Project Description:

Objectives: Hereditary information residing in the nucleotide sequences of deoxyribonucleic acid (DNA) is expressed via a certain class of ribonucleic acid molecules, "messenger-RNA". Cellular differentiation and control, at least in part, are reflections of messenger RNA populations which differ from tissue to tissue though a common genome is shared. Messenger RNA molecules possess base-sequence complementarity to those regions of a DNA molecule which were their templates at the time of transcription. Demonstration of this base-sequence complementarity is possible using isolated DNA, denatured to the single-stranded state, with which the single-stranded RNA molecules can hybridize. DNA-DNA hybrids can also be formed. Nucleotide sequences held in common between two sources of DNA or RNA, that is, regions of nucleic acid homology, are thus demonstrated. This search for nucleic acid homologies among tissues is a molecular approach to genotypic (DNA-DNA homology) and phenotypic (DNA-RNA homology) similarity.

The objectives of this project are a physico-chemical and biochemical study and comparison of the properties and homologies of the nucleic acid molecules of normal rat liver and a "minimal deviation" hepatoma, the Morris hepatoma 5123.

Methods Employed: Fractionation of cell components; differential, analytical, sucrose gradient and equilibrium ultracentrifugation; spectrophotometry; radioactive labelling; methylated albumin-kieselguhr columns for nucleic acid fractionation, Nygaard-Hall (and modification of Gillespie-Spiegelman) nitrocellulose membrane technique for hybridizing nucleic acids, isolation and purification of DNA-dependent RNA polymerase, in vitro synthesis on DNA and chromatin templates of complementary RNA, sodium borohydride procedure for in vitro labelling of ribonucleic acid.

Major Findings:

(1) Liver-Hepatoma Nucleic Acid Homology Studies. Rat liver chromatin has been prepared according to the method of Marushige and Bonner. This procedure yields a solubilized chromatin. Preparations appear to be reproducible; they are similar to Marushige and Bonner's as judged by UV spectrum, changed T_m and thermal denaturation profile as compared to purified liver DNA, and by V_{max} and K_m characteristics compared to purified DNA.

The rat liver chromatin was used as template for the in vitro synthesis of highly-labeled radioactive RNA. The RNA polymerase employed for the transcription of chromatin was prepared from E. coli B according to Chamberlin-Berg (Fraction IV) and assayed negatively for endogenous DNA. The tritium-labeled RNA synthesized from liver chromatin was hybridized to liver DNA in the presence and absence of unlabeled competitor RNAs isolated from rat liver and Morris hepatoma 5123. The membrane filter hybridization procedure, as modified by Gillespie-Spiegelman, was employed.

The results of the hybridization competition experiments were: (a) liver nuclear RNA competes more efficiently than hepatoma nuclear RNA at each level of competitor. At high competition levels of liver and hepatoma, only 8% and 24%, respectively, of the labeled RNA remains. The two curves suggest that liver nuclear RNA has a greater similarity than that of hepatoma to the in vitro synthesized RNA. Since the almost complete competition with liver nuclear RNA suggests (but does not prove) "fidelity" of the transcription of chromatin to that in vivo, these results may indicate in vivo differences in nuclear RNA populations between hepatoma and normal liver; (b) liver cytoplasmic RNA competes more efficiently than hepatoma cytoplasmic

RNA with the in vitro liver chromatin-transcribed RNA. A plateau level of binding corresponding to about one-half of the hybridized in vitro synthesized RNA, was found even with large amounts of hepatoma RNA. In contrast, liver cytoplasmic RNA was able at high levels to compete out completely the hybridized in vitro RNA. The plateau suggests an absence in hepatoma cytoplasm of RNA species present in the in vitro transcribed population, and by inference, in liver cytoplasm; this is in accord with recent observations by Drews et al., in an in vivo system with similar hepatomas. (c) If the liver nuclear and cytoplasmic RNA competition curves are compared from (a) and (b), the former is seen to compete more efficiently with the labelled RNA prepared on liver chromatin. This is not unexpected since a relatively larger amount of the cytoplasmic RNA is probably ribosomal and transfer RNA. At very high levels of liver cytoplasmic RNA all of the chromatin-produced RNA is displaced. This complete competition can be interpreted to mean that cytoplasmic RNA contains, at least in low amounts, all of the sequences which were transcribed on chromatin. This appears to be at variance with the results of others in which the transfer of only a limited number of nuclear RNA species to the cytoplasm was observed. Whether this is a consequence of our use of in vitro synthesized RNA or, for example, a higher than suspected contamination of our cytoplasmic preparations of RNA with nuclear RNA is under investigation.

(2) Liver Chromosomal RNA. Dr. Stanley Shackney has prepared rat liver chromatin and from it isolated by the procedures of Huang, Widholm and Bonner an RNA of low molecular weight. We are investigating whether this RNA has the properties and functions of the chromosomal RNA reported by Huang, Bonner et al., (e.g., Science 159: 47 (1968)). These workers have reported a new class of RNA,chromosomal RNA, which has a very high content of dihydrouridylic acid (27 mole-percent in pea buds), is small (about 40 nucleotides long), is bound to chromosomal protein, and which may very well provide the specificity of repression in higher organisms in differentiation.

Significance to Biomedical Research and the Program of the Institute: Careful comparison of DNA sequence homology of normal and tumor DNA would clarify the question whether the content of DNA information in the two tissues is equivalent or not. Messenger RNA molecules function as the carriers of genetic information from DNA to the sites of protein biosynthesis. They must, therefore, be intimately concerned with cellular processes of differentiation and control. Therefore, message differences found between RNA populations of neoplastic tissue and its tissue of origin should increase understanding of the neoplastic process.

Proposed Course of Project: Study of the nucleic acid homologies between rat liver and Morris hepatoma 5123 will be continued. The physico-chemical studies of liver and hepatoma nucleic acids, development of new techniques and measurements will be continued.

Use of isolated, purified nuclei and of tissue culture as well as the *in vitro* use of chromatin primers with RNA polymerase to prepare high specific activity messenger RNA will be investigated and continued. Isolation, properties and study of the function of chromosomal RNA will be continued. Also, fractionation of chromosomes from synchronized tissue culture-grown cells will be investigated.

Honors and Awards: None

Publications: None

Serial No. NCI-5519
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Possible Role of Mitochondrion and of the Mitochondrial DNA in Differentiation and in Neoplastic Transformation by Oncogenic Virus.

Previous Serial Number: None

Principal Investigator: Peter T. Mora

Other Investigators: Dr. Allan T. Leffler, II, Mrs. Ellen Creskoff, Mrs. Vivian McFarland

Cooperating Units: Dr. Robert Ting (C:LBGY - 3218) Laboratory of Biology, NCI, NIH.

Man Years (July 1, 1967 through June 30, 1968)

Total:	5
Professional:	2.5
Other:	2.5

Project Description:

Objective: The objective of the project is to determine what role, if any, the mitochondrion and the mitochondrial DNA (M-DNA) play in differentiation and in neoplastic transformation by oncogenic virus. To this end current work is directed to the preparation and characterization of M-DNA and M-RNA of high purity, from rat liver and kidney, and from a polyoma-induced rat tumor (Ting 3049).

Methods Employed: Liver and kidney mitochondria, isolated by an improved differential centrifugation technique, are subjected to SLS-phenol extraction of the nucleic acids. RNA is largely removed from the DNA by hydroxyapatite chromatography; where necessary the DNA is further purified by CsCl equilibrium buoyant density centrifugation.

For the tumor cells, grown in tissue culture, advantage is taken of the fact that M-DNA is over 50% soluble in 0.6% SLS, 1M NaCl at 0°C, while nuclear DNA (N-DNA) is largely precipitated. To effect further purification of M-DNA from residual contaminating N-DNA, the DNA (isolated and purified as above) is heated to 100° which results in a denatured single stranded DNA (SS-DNA), then incubated at 70°. After the incubation the M-DNA renatures to double stranded DNA (DS-DNA), while N-DNA does not. The DS-M-DNA is then separated from the SS-N-DNA with high temperature hydroxyapatite chromatography.

The M-DNA has been characterized by buoyant density and sedimentation velocity determinations in the analytical ultracentrifuge, spectral base analysis, DNA-DNA hybridization on nitrocellulose, strand separation, reannealing kinetics, and RNA-DNA hybrid formation detected on hydroxyapatite.

Results: Using the isolation techniques as finally worked out, it is possible to prepare M-DNA in 100-200 microgram quantities with a known level of N-DNA contamination. In vivo and in vitro labelling was done with ^{3}H-thymidine and $^{32}PO_4$; specific activities were 100-1000 times greater in vitro.

Our rat liver M-DNA bands very close or exactly on N-DNA in the CsCl gradient, suggesting equal average base composition, but on spectral base analysis M-DNA has a significantly higher average GC content. Either an abnormal base with possible glucosylation is shifting the apparent buoyant density or the known ring structure of M-DNA changes the spectral characteristics in a way not accounted for by the proposed equations used for calculation of the base composition. Experiments are in progress to determine which of these explanations is correct.

To determine the informational size of the M-DNA, the rate of renaturation was followed; that this rate is proportional to the genome size has been shown by a group at the Carnegie Institute of Washington. Early experiments give informational molecular weight estimates of 5-8 x 10^6 daltons, in good agreement with the known physical size of 9-10 x 10^6 daltons. This implies that the mitochondrion, like the bacterium, has a plural and variable number of identical genomes.

Experiments to measure the extent of nucleotide sequence homology between M-DNA's from two basically epithelial organs, liver and kidney, and between liver M-DNA and 3049 tumor M-DNA, are in a primitive state, as are experiments to examine the M-RNAs found in the two organs.

We have now demonstrated strand separation in an alkaline CsCl gradient. Incidentally this provides a more sensitive and reliable test for nuclear contamination than renaturation criteria: with pure M-DNA two peaks are observed with a buoyant density difference of 0.03 gm/cc. A contaminated preparation has a third peak midway between the two, corresponding to the position of pure N-DNA. More importantly it makes possible experiments to determine if one or both DNA strands are transcribed into RNA. Heretofore such work has been limited to viruses.

One observation has been repeatedly made in the course of this work: It is unfortunately not possible to predict beforehand the results of the purity test, either based on renaturation criteria or alkaline CsCl gradient behavior. Thus one should probably assume much of the prior work in the field was done on material with variable and unknown amounts (possible as high as 99%) of nuclear contamination.

Significance to Biomedical Research and the Program of the Institute: The genetic function of M-DNA is established in Neurospora and almost as well established in higher organisms as well. Thus there is a reservoir of genetic information outside the cell nucleus. To what extent it is independent of nuclear control and influence is presently unknown, but the successful completion of the experiments outlined, and presently underway, should give considerable evidence toward this matter.

Proposed Course of Project: We hope to establish which of the two strands of the M-DNA is actually transcribed to RNA, and determine its functional role in the cell vis à vis the nucleus and cytoplasm. By studying the effects of various antibiotics on the synthesis of M-DNA and RNA in tissue culture, we can possibly detect nuclear control of mitochondrial biogenesis, or the lack thereof. We also plan to conclusively demonstrate the absence (or the presence) of the viral genome in the mitochondrial DNA of malignant cells transformed by oncogenic DNA viruses.

Honors and Awards: None

Publications: None

Serial No. NCI-3103-B
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Study of the RNA of Avian Tumor Viruses.

Previous Serial Number: Same

Principal Investigator: Samuel W. Luborsky

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	0.9
Professional:	0.4
Other:	0.5

Project Description:

Objectives: To examine the infectivity of the RNA extract obtained from Rous sarcoma virus (RSV).

Many attempts have been made in the past to demonstrate infectious activity in the nucleic acid extracts of virus induced tumors, or in those of variously purified virus preparations. In general, these extracts have been little characterized. No report has yet been substantiated in the avian leukosis virus system.

In the past few years, methods have been developed for extracting the RNA from RNA-containing animal viruses, in presumably intact form. Physico-chemical characterization of such an RNA from RSV indicated that it is an intact molecule, since its molecular weight was large enough to account for all the RNA in the virus particle. This RNA should therefore provide the best material for successfully infecting a suitable tissue culture cell system, and thereby provide a new and valuable approach to the study of the molecular virology of infection. The information that would be provided by such a study is a necessity for any understanding of the processes of viral infection and transformation of cells.

The present study was undertaken to determine the ability of RSV RNA to infect chick embryo cells in tissue culture, to determine whether the genetic information in the intact RNA in a physically uninterrupted single molecule is sufficient for biological activity, i.e., (1) for directing the production of new virus particles, or (2) for inducing the neoplastic transformation of cells in tissue culture.

In other systems for which animal virus RNA infectivity has been demonstrated, various treatments of the virus-susceptible cells were usually required to permit the viral RNA to infect the cells. Substances such as, for example, DEAE-dextran, polyornithine, putrescine, cadavernine, or other polyamines, have been reported to permit or enhance the infectivity of poliovirus RNA in Hela cells, or of EMC virus RNA in Hela cells, when added either before or during the RNA inoculation period. Also, treatment of the cultures under hypertonic salt conditions has been reported effective. Actinomycin D was shown to enhance the infectivity of poliovirus RNA in Hela cells.

Major Findings: Based upon information obtained from a literature survey, a variety of these manipulations were performed on normal chick embryo cells, to determine what compounds and what types of treatments would be tolerated by these cells. Cell viability was tested by dividing the treated cultures and observing cell proliferation. In spite of the trauma experienced by these cells, if the cultures became confluent within 2-4 days, the treatment was considered "non-toxic". Cells were treated with actinomycin D, polyornithine, and bentonite, and acceptable concentrations and incubation times were determined for each, in both hypertonic and isotonic salt solutions. Similarly, non-toxic incubation conditions and concentrations were sought for the inoculation of the cells with an RNA solution containing DEAE-dextran and putrescine. It was decided to use these compounds together at this point since both had been reported to enhance poliovirus RNA infectivity, presumably by different mechanisms.

The effect of these compounds on the susceptibility of RNA to degradation was investigated in a model system, using commercial yeast RNA (1-2 mg/ml) and pancreatic RNase (.2 µg/ml). DEAE-dextran exerted a small protective effect on the RNA, which reached a plateau at DEAE-dextran concentrations above 500-700 µg/ml. Putrescine continued to provide increasing protection at the highest concentration examined, 0.01M. In agreement with the suggestion in the literature that the mechanism of action of the two substances is different, DEAE-dextran did not provide additional protection when used together with putrescine, but on the contrary, always reduced the degree of protection below that exhibited by the putrescine alone. At the concentrations of these

substances chosen for use in cell culture, significant protection of the RNA against enzymatic degradation is still evident in the model system studies.

RNA was extracted from a purified RSV preparation and was inoculated into chick embryo cell monolayers. Groups of these cells had been pretreated under the chosen conditions with actinomycin D and polyornithine in isotonic or in hypertonic solution, and washed with a low concentration of bentonite. The RNA inocula contained about 5 µg/ml viral RNA together with DEAE-dextran and putrescine, in either hypertonic or isotonic salt solution. After a 10 minute incubation period, polyornithine was added to the inoculated cell monolayers to further enhance the infectivity of the RNA, and the incubation was continued for another 30 minutes before adding growth medium. The cells were grown and subcultured as required, while evidence of RSV-transformed cells was sought. Some plates were lost at the outset to contamination - both positive and negative controls, as well as experimental plates - and some plates never recovered from their trauma. In separate experiments, the remaining cells grew poorly, especially after some subculturing, presumably because they were not originally a continuous culture line.

To test for the presence of virus progeny, after about 7-10 passages, the fluids from some of these cultures were inoculated into fresh chick cell monolayers. Virus particles produced by the presumptively infected cultures should be able to infect fresh cell cultures. These growing cultures should exhibit large vacuolated infected cells, usually growing in groups surrounded by a monolayer of normal uninfected smaller cells. In addition, fluids were also stored for more critical evaluation and quantitation of virus particles by the focus-forming assay for transformed cells under agar overlay.

Many cells were observed after the first few passages, which resembled RSV-transformed cells. Similar cells were noted also in plates inoculated with the fluids of the passaged cultures. However, these encouraging results must be considered as tentative until the quantitative focus-forming assays can be completed.

Significance to Biomedical Research and the Program of the Institute: If, as these preliminary results suggest, the RNA extracts are infectious, they then indeed contain RNA molecules intact enough to contain sufficient information to direct at least the production of progeny virus. The requirements for infectivity of the RNA can then be investigated, e.g., the size of the RNA required, the effect of various kinds of chain breakage, the effects of competition with other kinds of RNA, etc. In addition, the comments noted in the section on "Significance", Project NCI-5503, also apply to this work.

Proposed Course of Project: Focus-forming assays for RSV will be carried out on the fluids of these cultures. In addition, electron micrographs of cells from these cultures will be screened for the presence of RSV-like particles not present in the negative control plates.

Although the molecular weight of this viral RNA has been estimated indirectly from an empirical relationship, measurements will be made with future extracts to determine the molecular weight directly by sedimentation equilibrium studies.

Honors and Awards: None

Publications: None

Serial No. NCI-5503
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Properties of Mouse Leukemia Virus RNAs.

Previous Serial Number: Same

Principal Investigator: Samuel W. Luborsky

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	1.1
Professional:	.6
Other:	.5

Project Description:

Objectives: The objectives described for Project NCI-3103B apply equally to this work. Earlier procedures developed in this work enabled us to obtain undegraded RNA in an extract of the Rauscher leukemia virus, RLV, as determined by physicochemical methods. We wished (1-a) to investigate the degree of intactness of the RNA by another method, the ability to direct the production of progeny virions, (1-b) to determine whether the genetic information in the intact RNA is sufficient for this biological activity, and (2) to further characterize the size and shape of the RNA molecule obtained from RLV by more direct physical means. The previously estimated molecular weight was obtained indirectly, from the sedimentation coefficient, s, using an empirical relationship between s and the molecular weight of RNA molecules.

Major Findings: See Project NCI-3103-B, Major Findings section, for background to the determination of the proper treatment conditions for the cell monolayers prior to and during the inoculation of the RNA. Based upon the preliminary results obtained with the chick embryo cell system (Project NCI-3103-B) a continuous line of normal rat kidney cells, known to be

susceptible to RLV infection, was subjected to the various treatments mentioned in that report, to find appropriate conditions for recovering a sufficient number of viable cells after treatment. The "non-toxic" concentrations and incubation times found acceptable to these cells were similar to those used with the chick cell system, and involved pretreatment with actinomycin D, polyornithine, and bentonite, followed by inoculation of the RNA in the presence of DEAE-dextran and putrescine.

A pooled RLV preparation was purified on sucrose gradients, and its RNA extracted according to methods developed earlier in this project. Normal rat kidney cells were inoculated under the conditions chosen, and the cultures were divided as required. After 5-6 passages, some of the cells were prepared for electron microscopy, to look for virus-like particles. This work is in progress. Bioassays will be performed.

(2) A portion of the RNA extract was subjected to a variety of analytical ultracentrifugal techniques, using the ultraviolet absorption optical system with the photoelectric scanner to record the data. Sedimentation velocity showed the RNA extract to be quite similar to those previously obtained, containing 40-50% of a fast-moving 70S species. Sedimentation equilibrium studies using the Yphantis meniscus depletion technique were performed to provide a direct measurement of molecular weight. As expected for long, filamentous, high molecular weight polymers, the RNA exhibited very non-ideal thermodynamic behavior in these experiments (apparent molecular weight very low and strongly concentration dependent, deviation of concentration distribution from that predicted by simple theory). Therefore, measurements were required over a range of RNA concentrations. Data were also obtained to permit another independent calculation of the weight-average molecular weight by the Archibald approach-to-equilibrium technique. A diffusion coefficient will also be obtained from these calculations. The buoyant density of the RNA was determined by isopycnic sedimentation in a Cs_2SO_4 density gradient. An examination of this data at a few wavelengths will also permit an independent estimate of the lower limit of the molecular weight to be made.

Because of the possible instability of the RNA, these experiments were all performed in rapid succession to obtain satisfactory data before calculations of any of the results were undertaken.

Significance to Biomedical Research and the Program of the Institute: The comments contained in the section on "Significance" in Project NCI-3103-B apply equally here as well. This type of physical and biological characterization of murine leukemia viruses is fundamental to the characterization of the action mechanism of such viruses. The demonstration of the infectivity

of the isolated RNA of such viruses, and the correlation of this activity with their physical properties is a primary goal of molecular biology, necessary for a better understanding of viral multiplication and cell transformation by viruses. Much useful information as to the mechanism of action of viruses can be gained from such an approach. Information on the nucleic acids of murine leukemia viruses may be useful in aspects of studies on human leukemia, especially if similar viruses are demonstrated to be involved in the causation of human leukemia.

Proposed Course of the Project: (1) The fluids from the subcultured inoculated cells will be tested for helper activity with Moloney sarcoma virus. Those samples showing activity will be tested for the production of Rauscher disease in mice. The production of RLV-like particles from inoculated cells will be monitored by electron microscopy. Other preparations will be examined.

(2) The results of the physical measurements will be calculated. Other preparations will be examined. Better data will be obtained for the diffusion coefficient. The size and shape of the RNA molecule in solution will thus be determined.

Honors and Awards: None

Publications: None

Serial No. NCI-5512
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Formation of Transfer RNA-Ribosome-Template Complexes: The Structural Requirements for Transfer of Ribonucleic Acid for Interaction with Ribosomes.

Previous Serial Number: Same

Principal Investigator: Sidney Pestka

Other Investigators: None

Cooperating Units: None

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1/4
Professional:	1/8
Other:	1/8

Project Description:

Objective: The transfer RNA interaction with ribosomes during protein biosynthesis is a fundamental reaction in the translation of RNA codons. The purpose of this investigation was to evaluate the structural requirements of the tRNA molecules which are necessary for their interaction with 70S ribosomes and the individual ribosomal subunits.

Methods Employed: E. coli cultures, ribosomes, transfer RNA, E. coli extracts, aminoacyl-tRNAs, ribosomal subunits were prepared. Several tRNA fractions were prepared containing different numbers of bases deleted from the 3'-hydroxyl end of the molecule. These fractions were then tested for their ability to inhibit ^{14}C-Phe-tRNA binding to ribosomes and 30S subunits in the presence of a poly U template.

Major Findings: 1) Periodate treated tRNA appears to be inactive in binding to 70S ribosomes primarily because of extensive oxidative alterations rather than because of elimination of the terminal adenosine at the 3'-hydroxyl end of the molecule.

2) Removal of the terminal adenosine, the penultimate cytosine and the antepenultimate cytosine from the tRNA molecules converts those molecules into less active ones in their interaction with ribosomes in that order, but does not eliminate their ability to interact with ribosomes.

Significance to Biomedical Research and the Program of the Institute: This study is designed to determine requirements for tRNA interaction with ribosomes. Since tRNA is an important component of all cells and may be involved in cellular control of protein synthesis in both bacterial and mammalian cells, structural requirements of tRNA for interaction with ribosomes should be understood. The present results help to elucidate this as well as to clarify spurious results in the literature.

Proposed Course of Project: The effects of antibiotics and other agents on tRNA interactions with ribosomes are being evaluated. In addition, the role of 5S RNA in protein synthesis is being examined.

Honors and Awards: None

Publications: None

Serial No. NCI-5515
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A new assay for codon recognition and interaction of transfer ribonucleic acid with 50S subunits.

Previous Serial Number: None

Principal Investigator: Sidney Pestka

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	1/2
Professional:	1/3
Other:	1/6

Project Description:

Objective: The purpose of this investigation was to evaluate the nature of the interaction between tRNA molecules and ribosomes as well as to determine which portions of the tRNA molecules are involved. Furthermore, an assay independent of binding of ribosomes to cellulose nitrate filters was sought for translation of RNA codons.

Methods Employed: E. coli cultures, ribosomes, transfer RNA, extracts and aminoacyl-tRNA were prepared as previously described in this report. Large amounts of ribosomal subunits were prepared by zone centrifugation techniques.

Major Findings: 1) Aminoacyl-tRNA bound to 70S ribosomes in the presence of a template was resistant to digestion by pancreatic ribonuclease. Based on this observation, a convenient sensitive assay for codon recognition and aminoacyl-tRNA binding to ribosomes was developed.

2) The protection of tRNA from digestion by RNAse occurred as a result of the association of aminoacyl-tRNA with the 50S subribosomal particle and thus the assay provided a sensitive functional test involving the specific interaction of tRNA with the 50S subunit of E. coli ribosomes. The kinetics of attachment of 50S subunits to 30S ribosome-polyribonucleotide template-aminoacyl-tRNA complexes was studied by the assay.

Significance to Biomedical Research and the Program of the Institute: This study is part of an effort devoted to examining cellular control of protein biosynthesis. The tRNA-ribosome-template interactions are one possible level of control of protein synthesis. The assay developed provides an additional technique for evaluating the formation of these ternary complexes. Since the attachment of 30S and 50S subunits and messenger RNA templates appears to involve individual steps during the initiation of protein biosynthesis in cells, the method provides a rapid and sensitive procedure for studying the attachment of 50S subunits to the complex. Furthermore, the assay provides an additional parameter for evaluating the site of action of antibiotics and other substances interfering with protein biosynthesis.

Proposed Course of Project: The rate and nature of attachment of 50S subunits to the 30S-template-tRNA complex is being studied with the hope of elucidating the nature of the 50-30S interactions as well as the interaction of tRNA with the 50S subunit. The influence of various antibiotics on the attachment of 50S subunits is being examined, with the hope of elucidating their mechanism of action. In addition, the 50S ribosomes are being fractionated into protein and RNA sub-fractions. These 50S sub-fractions are being utilized to determine the requirements for the interaction of 50S particles with tRNA and 30S subunits.

Honors and Awards: None

Publications: Pestka, S.: Studies on the formation of transfer ribonucleic acid-ribosome complexes. IV. A new assay for codon recognition and interaction of transfer ribonucleic acid with 50S subunits. J. Biol. Chem. Submitted for publication.

Serial No. NCI-5516
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of Formation of Peptide Bonds.

Previous Serial Number: None

Principal Investigator: Sidney Pestka

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	1 1/2
Professional:	1
Other:	1/2

Project Description:

Objective: Protein biosynthesis consists of a vast number of coordinated and cooperative reactions which proceed in an orderly sequence and which perhaps may be biochemically coupled. The formation of the peptide bond is one step in this process and its elucidation was the purpose of this investigation.

Methods Employed: E. coli cultures were grown and harvested by standard microbiological methods. Ribosomes, transfer RNA, E. coli extracts and aminoacyl-tRNA synthetases were prepared. Aminoacyl-tRNA binding to ribosomes was studied by procedures previously published. Chromatographic methods were developed to separate phenylalanine and the series of oligophenylalanine.

Major Findings: 1) Peptide bond formation from aminoacyl-tRNA and ammonium chloride-washed ribosomes was found to occur extensively in the absence of supernatant enzymes. Although diphenylalanine was the major product, some tri- and tetraphenylalanine was detected.

2) Oligophenylalanine synthesis in the absence of supernatant factors (peptide bond synthesis) and amino acid polymerization

into long polypeptides in the presence of supernatant (protein synthesis) were found to differ in magnesium and temperature optima and other characteristics; GTP is not required for the former as it is for the latter; the sulfhydryl inhibitor para-chloromercuribenzosulfonate has little effect on the former while completely abolishing the latter. In addition an absolute requirement for ammonium or potassium ions was shown for peptide bond synthesis.

3) The results suggest that peptide bond synthesis from aminoacyl-tRNA is the property of ribosomes and is not mediated by supernatant protein factors.

Significance to Biomedical Research and the Program of the Institute: This study is a part of an effort which is directed at examining protein synthesis. The formation of tRNA-ribosome-template interactions are one step of protein synthesis. The present study indicates that during the formation of these complexes oligopeptides are formed. Peptide bond formation from aminoacyl-tRNA appears to be an integral property of the ribosome itself.

Proposed Course of the Project: The kinetics of peptide bond synthesis as well as the influence of various antibiotics on the process is being evaluated. Furthermore, the diphenylalanine-tRNA-ribosome-template complex is being utilized as a substrate to study the processes of translocation during protein synthesis. The formation of triphenylalanine-tRNA is being utilized as an assay for translocation. The dependence of translocation on various supernatant and other factors (as the transfer factors and GTP) is being examined. Isolation of the peptide synthetase located on the 50S particle is in progress.

Honors and Awards: None

Publications: Pestka, S.: Studies on the formation of transfer ribonucleic acid-ribosome complexes. III. The formation of peptide bonds by ribosomes in the absence of supernatant enzymes. J. Biol. Chem. 243: 1968 (In press).

Serial No. NCI-5517
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Control Mechanisms in Normal and Cancer Cells.

Previous Serial Number: None

Principal Investigators: Sidney Pestka; Robert Gallo, NCI-3406; Seymour Perry, NCI-3406.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	2
Professional:	1
Other:	1

Project Description:

Objective: Control of cellular growth, differentiation and carcinogenic transformation may be mediated by control of protein biosynthesis. Alterations of transfer RNA levels by cells provide a feasible means of controlling protein biosynthesis and consequently enzymatic and structural cellular components. A major purpose of this study is to determine the levels of specific transfer ribonucleic acid species in both normal and cancer cells.

Methods Employed: Normal and leukemic leukocytes from human donors are isolated by standard techniques. Transfer RNA and aminoacyl synthetases are prepared from each purified cellular fraction by methods previously published. Transfer RNA fractions from comparable normal and leukemic cells are acylated with a specific amino acid using homologous synthetases. The combined mixtures of ^{14}C- and ^{3}H-aminoacyl-tRNA prepared from the leukemic and normal cell tRNA, respectively, is then analyzed to detect differences between the ^{14}C- and ^{3}H-aminoacyl-tRNA patterns. Presumably, any differences between the carbon-14 and tritium

patterns reflect differences between normal and leukemic cell tRNA. Where differences are localized, tRNA and acylation are examined in detail. Computer computation of the results with double labelling tracer techniques are employed.

Major Findings: The methods and studies planned are being initiated at the present time.

Significance to Biomedical Research and the Program of the Institute: This study is designed to determine if differences in transfer ribonucleic acid between normal and cancer cells exist. In addition we hope to determine if transfer ribonucleic acid alterations in cancer cells can account for some of their oncogenic properties.

Proposed Course of Project: All twenty aminoacyl-tRNA families will be examined by the methods described in both normal and cancer human cells of various types. In addition to studying normal and leukemic white cells, other cancer cells will be examined. We plan to evaluate the effects of various therapeutic agents on the aminoacyl-tRNA levels in cancer cells and to correlate the results with therapeutic response.

Honors and Awards: None

Publications: None

Serial No. NCI-5507
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Nature of Large Molecular Weight Forms of β-Galactosidase in Escherichia coli K12.

Previous Serial Number: Same

Principal Investigator: Sidney Shifrin

Other Investigators: Dr. Edward Steers, Jr., and Dr. Sally Marchesi, LCB, NIAMD, NIH.

Cooperating Units: Laboratory of Chemical Biology, NIAMD, NIH.

Man Years (July 1, 1967 through June 30, 1968)

Total:	1 1/2
Professional:	1 1/2
Other:	None

Project Description:

Objective: To examine the nature of the interactions involved in maintaining structure of large molecular weight aggregates with β-galactosidase activity isolated from Escherichia coli K12 strain 3300.

Methods Employed: In addition to purification methods outlined in a previous report, the multiple forms have been examined by free boundary electrophoresis and by electron microscopy.

Major Findings: The large aggregates of β-galactosidase with sedimentation coefficients of 45S, 33S, 28S and 23S also have different mobilities in electrophoresis. These different forms have been separated and spread for electron microscopic examination. In addition to long thin strands of subunits, we have found hexamers in hexagonal array and octamers in octagonal array. These forms are not interchangeable nor can they be formed in vitro from the major tetrameric aggregate.

Significance to Biomedical Research and the Program of the Institute: The specific interaction of protein subunits is and example of macromolecular interactions which participate in the control of living systems. Study of these forces *in vitro* is useful for a better understanding of these control processes *in vivo*.

Proposed Course of Project: This project is being terminated.

Honors and Awards: None

Publications: None

Serial No. NCI-5518
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Transferase Activity of β-Galactosidase from Escherichia Coli K12.

Previous Serial Number: None

Principal Investigator: Sidney Shifrin

Other Investigator: Gilbert S. Hunn

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	2 1/4
Professional:	1 1/4
Other:	1

Project Description:

Objective: To study the enzymatic activity of β-galactosidase in the presence of low molecular weight alcohols: n-propyl-, isopropyl alcohols, ethanol and methanol.

Methods Employed: The wild type strain of E. coli K12 (strain 3300) was grown in a 300 liter fermenter and the bacteria were harvested during logarithmic growth. The bacterial cells were broken by a French pressure cell and the enzyme was purified on Sephadex G-200 and DEAE-cellulose. Further purification to the 16S tetramer was carried out by sucrose density centrifugation. The fractions were examined on disc electrophoresis stained for protein with amido Schwartz and examined for enzymatic activity. The enzymatic activity was studied kinetically and the protein was followed by analytic ultracentrifugation methods, ultraviolet spectroscopy and fluorescence spectroscopy. Reaction products were separated chromatographically and were examined by carbohydrate chemical methods.

Major Findings: Purified β-galactosidase (16 S tetrameric aggregate) was stimulated in its ability to cleave the synthetic substrate ortho-nitrophenyl-β-D-galactopyranoside by low concentrations of the short chain monohydroxy alcohols. Up to concentrations of 1.5 M, n-propyl alcohol enhanced the activity nearly threefold (as measured by the release of o-nitrophenol) and so did to a lesser extent ethanol, and for the least extent methanol. At 1.75 M concentrations this order is reversed and methanol is most effective as a stimulant up to concentrations of 10 M at which point the activity is still 1.5 times its normal rate. In n-propyl alcohol, at concentrations of 2.5 M or above, however, the enzyme precipitates and is inactivated.

Previous studies had suggested that the subunits of the active tetramer of the enzyme are in part held together by hydrophobic forces. It was, therefore, our hypothesis that increasing hydrophobicity as would be found with longer alkyl alcohols should also dissociate the enzyme. Analysis by sedimentation velocity in the presence of the alcohols showed that the subunits remain associated in their tetrameric form. In addition there was no evidence that the alcohols induced any conformational changes in the protein as followed by ultraviolet difference spectroscopy and fluorescence ultraviolet spectroscopy.

That the alcohols do, indeed, disrupt hydrophobic bonds was demonstrated by the effect of the alcohols on the rate of thermal inactivation. At 47°C, the activity of β-galactosidase is lost most rapidly when heating occurs in the presence of n-propyl alcohol. The rate of thermal inactivation decreases in the following order: isopropyl alcohol, ethanol, methanol.

Kinetic analysis of the effect of the alcohol indicates a strong dependence on the presence of substrate for inactivation. Thus, in 10% n-propyl alcohol, the enzyme is activated at high concentration of substrate, but is inhibited by the alcohol at lower substrate concentrations. It appears that the alcohols must denature the protein in the absence of substrate although the structural changes are too subtle for detection by the methods we have employed. Further kinetic analyses in the presence of increasing concentrations of methanol indicates that the mechanism is of the sequential type (according to Cleland's designation); i.e., that ONPG and alcohol must bind to the enzyme before product is produced.

The products in the presence of alcohols were determined by paper chromatography and were found to include o-nitrophenol, galactose and alkyl-β-galactosidase where the alkyl group is derived from the alcohol. Thus, β-galactosidase functions as a transferase in addition to its hydrolase activity. In other words ONPG may be split by alcoholysis as well as hydrolysis.

Significance to Biomedical Research and the Program of the Institute: The enzyme β-galactosidase has served in the past to a great extent to elucidate the mechanism of enzyme induction. The ability of the enzyme to utilize hydroxy compounds other than water to cleave its substrate may be of extreme important in the metabolic control of enzyme function.

Proposed Course of the Project: One purpose of the project was to determine the nature of the forces which hold the subunits together. Studies with the alcohols revealed no effect on the aggregation of the subunits. Many different type of forces could participate beside hydrophobic forces, but full exploration of these forces would be a very lengthy project. The experimental observations have been submitted for publication.

Due to change of overall orientation of the senior investigator towards studying the molecular basis of cellular interaction in differentiating systems, these in vitro studies on galactosidase are being terminated.

Honors and Awards: None

Publications: Shifrin, S., and Hunn, G. S.: Transferase Activity of β-Galactosidase. J. Biol. Chem. Submitted for publication.

Serial No. NCI-5505
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the Factors Influencing Contact Inhibition of Cell Division of Mammalian Cells in Tissue Culture.

Previous Serial Number: Same

Principal Investigator: Peter T. Mora

Other Investigators: Mrs. Vivian McFarland, Dr. Vincent T. Marchesi, C:MBS; Dr. Richard Cornell, C:LBGY; Dr. George Todaro, C:ET; Dr. Roscoe Brady, Jr., N:LNC.

Cooperating Units: Dr. Ward Pigman and Dr. Saiyid Rizvi, New York Medical College, New York, New York.

Man Years (July 1, 1967 through June 30, 1968)

Total:	2.5
Professional:	1
Other:	1.5

Project Description:

Objective: To define and characterize the many factors influencing contact inhibition of cell division, or density dependent inhibition of cell growth, in tissue culture. To study the effects of cell surface membranes, fractions of these membranes and exudates of cells under dense growth conditions. To characterize the mucopolysaccharides and glycolipids in the surface membrane as well as those released into the medium by normal cells grown in tissue culture, and to compare these with the corresponding transformed neoplastic cells.

Methods Employed: Standard tissue culture techniques were used to study the growth of cells in tissue culture and the formation of monolayers in plastic petri dishes. The cells used were the highly contact inhibited 3T3 cells from a line of mouse embryo

fibroblasts isolated by Todaro and Green, and the SV 40 and polyoma virus doubly transformed variant SV-PY-3T3 cells which lost their contact inhibition but do not produce virus particles. Also C3H/HeN mouse cells grown in the tissue culture section, Laboratory of Biology in either horse serum (neoplastic cells) or fetal calf serum (non-neoplastic cells) were compared in some experiments.

Measurements of growth rate, confluent cell density of the monolayer, and rate of increase in cell density after confluence were made by direct cell counts, in a hemocytometer, of the trypsinized suspended cells, by measurement of the incorporation of tritiated thymidine into DNA after continuous or pulse labeling of the culture, or by counting the cells on the plate in situ using a calibrated eyepiece grid. Both liquid scintillation counting and autoradiographic techniques were used after tritiated thymidine labelling to identify the extent and location of actively dividing cells.

A special technique was developed for growing a monolayer of cells on both sides of an ultra-thin millipore filter. The technique was extended to allow simultaneous autoradiographic studies of the cells on both sides of the membrane. This made possible the quantitation of both cell density and incorporation of tritiated thymidine into the dividing cells on each side of the membrane using the phase microscope and calibrated eyepiece grid.

Electron microscopy, glycolipid separation, analysis of the carbohydrate and polysaccharide components, etc., were carried out by standard techniques.

Major Findings: Non-dividing mouse embryo fibroblasts (3T3 cells) which grew to a confluent cell density on one side of an ultra-thin millipore filter did not inhibit the active multiplication of the same type of cells growing at low cell density on the other side of the filter directly opposite the confluent side. The close proximity of the cells across the filter was not sufficient to cause inhibition of cell division. The phenomenon of "contact" or "density dependent" inhibition of cell division is therefore not mediated by a cellular exudate which remains concentrated near the cell surface, but likely by direct contact of the cell surfaces.

The degree of contact inhibition of cell division was correlated with the local cell density on the same side of the filter. This relationship was found to be influenced strongly by the supporting surface on which the cells were growing.

Next, we asked whether the inhibition of cell division could be attributed to the inert chemical composition of neighboring cell surface, that is, whether the residues of lyophilized cells retained the ability to inhibit the division of normal cells. In addition, we wanted to know whether cells in which DNA synthesis was impaired by irradiation would retain the capacity to effectively inhibit normal cells.

To answer these questions, confluent and non-confluent layers of 3T3 cells were prepared in tissue culture dishes and the cells were either lyophilized or irradiated in situ. Fresh 3T3 cells were then added to these prepared layers and their growth was followed using radioactive label. There was no growth of added cells on the confluent monolayers of either untreated or irradiated cells. Growth was unimpeded on the monolayers of morphologically apparently intact lyophilized cells. When cells were added to non-confluent cultures of either normal or irradiated cells the added cells grew until they had covered the remaining surface of the culture dish and had come into contact with the pre-existing cells. Thus, cells in which DNA synthesis was impaired by irradiation (but in which most other metabolic functions were preserved) continued to effectively inhibit, upon cell surface contact, the growth of normal neighboring cells. However, continuance of some metabolic function is essential since the lyophilized cell layer was not inhibitory.

To detect likely specific chemical changes in the cell surface, 3T3 cells and the transformed variant SV-PY-3T3 cells now are being compared in two ways. One approach (with Dr. Brady) is designed to detect changes in the glycolipids which are part of the cell surface. Preliminary results on cultures labelled with tritiated N-acetylmannosamine, which is a precursor of the ceramide glycosides, indicates that in the transformed cells at least two new ceramide glycosides (possibly sialic acid containing hematosides) are synthesized, which are absent in the normal 3T3 cells. This would be in line with the observed increase of net electronegativity of cell surfaces in neoplastic transformation.

In another approach the acid mucopolysaccharides released from the surface of the above cells are being compared (with Drs. Pigman and Rizvi). This work is still in the early methodological phase. Large amounts of interfering other macromolecules in the nutrient broth (serum, etc.) makes it difficult to use the existing techniques for the quantitative determinations of acid mucopolysaccharides. The major carbohydrate components released from 3T3 cells were identified as fucose, hexosamine and galactose.

Various electronmicroscopic techniques are under development (with Drs. Marchesi and Cornell) to compare the normal and

malignant cell surfaces, both when the transformation was initiated by viruses, and also when the tissue culture condition appear to influence the neoplastic transformation, as observed by Dr. Evans and her collaborators.

Significance to Biomedical Research and the Program of the Institute: It is important to study the role of cell surfaces in the intercellular control mechanisms which are responsible for contact inhibition of cell division, and to compare normal and neoplastic cells in this respect. This knowledge is also essential to our better understanding of the complicated cellular interactions which occur in normal embryogenesis and in maintaining the stability of normal differentiated tissues.

Proposed Course of Project: All the above outlined approaches, and collaborative undertakings will be continued and intensified to characterize the chemical and ultrastructural changes in cell surface which accompany changes in cell growth in neoplastic transformation. Studies on cell surface changes in embryonic differentiation are also being initiated.

Honors and Awards: None

Publications:

Schutz, L., and Mora, P. T.: Growth of Mouse Fibroblasts in the Presence of Irradiated and Lyophilized Monolayers of the Same Type of Cells. J. Cell. Physiol., 1968 (in press).

Schutz, L., and Mora, P. T.: The Need for Direct Cell Contact in "Contact" Inhibition of Cell Division in Culture. J. Cell. Physiol., 1968 (in press).

Serial No. NCI-5513
1. General Laboratories and Clinics
2. Macromolecular Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isolation and Characterization of Protein Components of Red Cell Membranes and Membranes of Cells in Tissue Culture.

Previous Serial Number: Same

Principal Investigator: Vincent T. Marchesi

Other Investigators: Dr. Edward Steers, Jr., NIAMD-LCB-19; Dr. Thomas Tillack, Laboratory of Experimental Pathology, NIAMD.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	3.5
Professional:	2.5
Other:	1

Project Description:

Objectives: To study the physical, chemical and biologic properties of spectrin, a fibrous protein which we have isolated from the membranes of red blood cells. We hope to determine the molecular structure and chemical properties of this protein after isolation from human red blood cells in order to determine how it is organized in the intact membrane and what its functional role might be. Such studies will also include attempts to isolate and characterize a similar protein from the surface membranes of other animal cells and from tumor cells.

Methods Employed: Membrane fractions are prepared from red cells by an osmotic lysis procedure; they are extracted by several different procedures and as a result some of the membrane bound proteins are dissociated from the lipid complex of the membrane and appear in solution in aqueous buffers. Extraction media include ATP, β-mercaptoethanol, EDTA, and, in certain experiments,

aqueous pyridine. The soluble proteins are concentrated by salt precipitation or pressure dialysis and further purified by column chromatography using Sephadex and Sepharose gels.

The purified proteins are further studied by electron microscopy using negative staining techniques and metal shadowing; polyacrylamide gel electrophoresis; free-boundary electrophoresis; analytical ultracentrifugation, and various immunoprecipitin reactions.

The proteins are further characterized by amino acid analysis, end group analysis, and peptide maps.

Major Findings: By subjecting isolated membranes of red blood cells to various extracting media made up of low ionic strength buffers, chelating agents, and β-mercaptoethanol we have solubilized approximately 25% of the total membrane-bound protein. This protein appears to be homogeneous by several physical criteria, and has the capacity to polymerize into helically coiled polymers which are identical in their ultrastructural appearance to actin, a fibrous protein of muscle. We established that this protein extracted from red cells was a specific component of the red cell membrane by showing that antibodies directed against this material did not react with serum protein or proteins released from red cells during hemolysis. We also found that antibodies against the membrane protein did not react with purified preparation of actin extracted from the same animals. We further showed that the ultracentrifuge and electrophoretic properties of the membrane protein also differed from those of muscle actin.

As a result of these studies we concluded that the protein extracted from the red cell membrane was a new species specific to the membrane and have named this component Spectrin (Science 159: 203, Jan. 1968).

Further studies of spectrin have shown that this protein does not have any bound lipid, carbohydrate, or nucleic acid, and appears to be a polymer unit in the native state. We have found that spectrin is present in all species of red blood cells so far examined (human, horse, rabbit, guinea pig, sheep and rat) and the proteins extracted from each species have the same electrophoretic properties. Also antibodies directed against guinea pig spectrin cross-react with spectrins extracted from cells of all the other species.

Preliminary amino acid analyses of spectrin extracted from several species also show that the total composition of these proteins is strikingly similar.

We have attempted to determine where spectrin is located in the intact membrane by studying extracted membrane preparation by electron microscopy. Studies of intact membrane indicate that a network of fibrous material forms part of the inner surface of the membrane in all species studied. After spectrin is extracted from membranes this fibrous network is no longer visible. Additional evidence that spectrin is present on the inside surface of the membrane is provided by studies using anti-spectrin antibodies. The results of such experiments also indicate that the antigenic sites on spectrin are not exposed to the outside surface of the cells. On the basis of these studies we have provisionally concluded that spectrin forms a fibrous network on the inner surface of the intact membrane.

During the course of these studies we became aware of a new method for the extraction of membrane protein which involved the use of aqueous pyridine.

By using pyridine after spectrin had been removed from membrane fragments by our earlier method we have been able to extract some new protein components of the red cell membrane. These new proteins are of particular interest since they contain a major portion of the carbohydrate of the membrane and all of the sialic acid. This is of some interest to us since there are many reasons for believing that this protein is located on the outside surface of the membrane. It seems likely that this sialic-acid containing protein may be important in determining surface specificity.

Experiments are now in progress to determine the chemical properties of this protein and how it is related to spectrin and to other components of the membrane.

Significance to Biomedical Research and to the Program of the Institute: As a result of our studies using the red cell membrane we are now in a position to apply the same methods to studies of the protein components of membranes of nucleated cells.

Thus we now have some specific approaches which we can use to explore the surface properties of mammalian cells in our search to determine what chemical properties of cells are concerned with surface recognition and other cell-cell interactions. In addition to their relevance to future studies of malignant cell surfaces these findings may also suggest ways to study the chemical basis for certain diseases of red blood cells such as hereditary spherocytosis.

Proposed Course of Project: We hope to continue using the red cell membrane system as a basic experimental model in order to develop methods for extraction and purification of membrane

components. In addition we now feel ready to apply some of the methods described above to other cell types and plan to start isolating and extracting cell membrane preparation of tissue culture cells.

We shall also continue with more detailed studies of the properties of spectrin and hope to determine its molecular weight, subunit structure, and certain aspects of its chemical composition.

We are also planning a detailed study of the sialic acid containing protein. This will involve developing methods for its purification. Since we feel that this protein or proteins may be involved in cell surface specificity as soon as we develop appropriate methods we will proceed directly to the study of its properties and distribution in cultured mammalian cells.

Honors and Awards: None

Publications:

Marchesi, V. T., and Palade, G. E.: The localization of Mg-Na-K-activated adenosine triphosphatase on red cell ghost membranes. J. Cell Biol. 35: 385-404, Nov. 1967.

Marchesi, V. T., and Steers, Jr., E.: Selective solubilization of a protein component of the red cell membrane. Science 159: 203-204, Jan. 1968.

SUMMARY STATEMENT
ANNUAL REPORT
July 1, 1967 through June 30, 1968
LABORATORY OF BIOCHEMISTRY

CYTOCHEMISTRY SECTION

Dr. D. Burk and Dr. M. Woods are examining the occurrence and function of a stress-modifiable anti-insulin hormonal system regulating glucose utilization in cancer and normal cells and its relation to chemotherapy at normal and hyperthermic temperatures. They have found that the rates of glycolysis (aerobic and anaerobic) in C_3H and $C_3H\text{-}A^{vy}$ mouse spontaneous mammary tumors (Heston) vary significantly even when taken from the same individual host mouse. In all of the individual mammary tumors tested, however, insulin stimulated and anti-insulins (Δ^1-testolo-lactone, testosterone, estradiol, Podophyllum fraction CC7E) inhibited glucolysis. Respiration, the Pasteur effect, and glycolysis in these mammary tumors were considerably more resistant to in vitro hyperthermy (43°-45°C) than in normal kidney, Ehrlich ascites cancer cells, or S91 melanoma. Agents which depressed glucose metabolism enhanced susceptibility to hyperthermic death whereas agents which increased the rate of glucose utilization produced the opposite effect. Uncoupling agents, or prior exposure to anoxia in the absence of glucose, greatly decreased cell resistance to subsequent hyperthermy. A prime objective is to find agents and/or procedures which will synergize with moderate hyperthermy (e. g., 40°-42°) in selectively killing cancer cells without destroying irreplacable normal tissues. Further studies of the glucolytic properties of tissue culture cell lines and clones continued to emphasize the importance of increases in glucolytic capacity (increased $Q^{N_2}_{CO_2}$ and decreased susceptibility to anti-insulin restraint) in the initiation and progression of malignancy.

They are also studying the combined actions of vitamins, peroxides, anti-catalases and moderate hyperthermy on cancer metabolism and growth compared to the respective normal cells of origin. Cancer cells display characteristically harmed respiration, increased glucolytic capacities, and usually much lower catalase activities. Many cancers are more susceptible to injury from hydrogen peroxide (H_2O_2) than are most normal tissues. Current and planned experimentation with possible intracellular H_2O_2 generators (vitamin C, riboflavin, K-vitamins, methylene blue, etc.) seeks to determine whether selective inhibition or killing of cancer cells can be obtained with such agents, and even more so when used in conjunction with catalase inhibitors such as the relatively non-toxic aminotriazole, or copper ion. Results obtained to date with e. g., sodium ascorbate and amino-triazole, with Ehrlich ascites tumor cells indicate that such model chemotherapeutic systems work effectively and synergistically in vitro. Corresponding in vivo

tests are under way with mice. High energy radiation (e. g., x-ray) and/or exposure of the cancerous tissue to moderate hyperthermy provide adjunctive treatments that may improve the over-all therapeutic results. With respect to hyperthermy, studies are also directed towards discovering procedures and agents which will not only selectively increase the thermal susceptibility of the cancer cells, but will also selectively increase the thermal resistance of the host.

NUTRITION AND CARCINOGENESIS SECTION

Dr. Harold P. Morris, one of the original members of the National Cancer Institute, has retired after 38 years of research in the Federal Service with over 30 at the National Cancer Institute.

During the past year he has continued his extensive biological and biochemical studies of primary and transplantable tumors with the collaboration of some 40 investigators in the United States and throughout the world. Animals bearing the various tumors were produced by Dr. Morris under a contract with Melpar, Inc., Falls Church, Virginia (PH-43-66-51). Emphasis has been placed on the induction of new tumors by chronic feeding of low levels of carcinogens, and on insuring the continued availability of large numbers of rats bearing different lines of "minimal deviation" hepatomas. A hepatoma tumor bank has been started. Dr. David R. Meranze, has, on a consultant basis performed pathological examinations of selected tumor tissues.

Additional transplantable primary hepatomas, have been induced by non-liver-specific carcinogens and several histologic types have been recognized. In the hepatoma systems it was found (Lea, Morris and Weber) that ^{14}C-thymidine incorporation into DNA closely paralleled the growth rate of hepatomas and thus provides another way of measuring growth. An extension of this study to transplantable rat kidney tumors was made with similar results. Collaborative studies (Dr. Nowell) of chromosomes in 41 hepatomas revealed that only 2 hepatomas had both the normal diploid number and normal karyotype although five other hepatoma lines were diploid but had some minor alterations in their karyotypes. One fast growing tumor was hypodiploid. Several slowly growing tumors were hyperdiploid and or aneuploid. Defective control of lipogenesis and/or fatty acid synthesis has been found by Elwood and Morris, and Sabine, Abraham, and Morris independently in five different transplantable hepatomas. The dietary regimens that greatly altered metabolic and enzymic activities in host or control livers were found not to affect enzymic activities of hepatomas. This finding may indicate a functional aberration that is fundamental to the neoplastic process.

Comparison of the responses of enzyme activity to increased dietary protein in host liver or normal liver, in collaboration with Potter, Pitot and Watanabe, led to the conclusion that the hepatoma enzyme activity tends

to be either "non-induced", "derepressed", or less sensitive to environmental factors than normal liver. Marked oscillatory patterns were noted for α-ketoglutarate transaminase in both host-liver and hepatomas, but serine dehydratase, glucose-6-phosphate dehydrogenase and citrate cleanage enzyme did not show cyclic changes in either host liver or hepatomas. Glycogen levels varied considerably. There appeared to be a reciprocal relationship between the activities of serine dehydratase and glucose-6-phosphate dehydrogenase. Both these enzyme activities from various tumors fall well within the range for normal rat liver under the same environmental conditions and the hepatomas tend to maintain these activities within the range of values of normal liver.

In the kidney tumor systems, three transplantable kidney tumors were developed by feeding diets containing 4'-fluoro-4'-biphenylacetamide. Each of the three kidney tumors had a different growth rate, the fastest growing line requiring about 3 months to grow to a useful size, whereas the slowest growing line required 12 months to grow to a similar size. Metabolic studies of the fastest and slowest growing kidney tumor lines revealed (Lea, Morris and Weber) increased activity of the glycolytic enzymes, hexokinase, phosphofructokinase, and pyruvate kinase. The activity of the gluconeogenic enzymes, glucose-6-phosphate and fructo-1,6-diphosphatase were decreased. Lactate production from glucose was very low or undetectable in fortified homogenates of rat kidney cortex but in the fastest growing kidney tumor there was a marked lactate production from glucose. The glucose-6-phosphate and 6-phosphogluconate dehydrogenase activities were increased. Thymidine-2-^{14}C-incorporation of DNA was greater for the fastest growing kidney tumor than for the slowest growing kidney tumor. The increases in the ratios of glycolytic to gluconeogenic enzymes in kidney tumors and the rise in thymidine incorporation, resemble the alteration previously observed in hepatomas and thus further emphasizes the similarity of neoplastic tissues.

Collaborative studies of kidney tumors undertaken with Dr. Katunuma have disclosed that the rapidly growing rat kidney tumor contained high activities of the phosphate-independent and also the phosphate-dependent glutaminase isozymes. The specific activity of the phosphate-dependent isozyme was 5 times higher in the tumor than in the normal kidney. Phosphate-independent glutaminase isozyme of liver and brain was activated by N-acetyl glutamate in contrast to the isoenzyme from normal kidney. The latter, however, was strongly activated by a peptide fraction isolated from kidney, but the liver and brain phosphate-independent glutaminase isozyme was not. However, the kidney tumor phosphate-independent glutaminase isozyme was activated by both N-acetyl glutaminate and the kidney peptide fraction. It thus seems that in this kidney tumor line two isozymes are not involved but that a single isozyme contains the binding sites for both activators.

The relative isozyme distribution of aspartate aminotransferase, alanine aminotransferase and L-malate: NAD oxidoreductase has been studied in hepatomas by Dr. T. Otani under conditions such as adrenalectomy and hormone administration. With alanine aminotransferase he has found that the anionic isozyme appears to be decreased or absent from the hepatoma. No marked differences have so far been found in the L-malate: NAD oxidoreductase system.

In his examination of "unnatural" amino acids, Dr. Otani has synthesized a number of β-hydroxy-α-amino acids and their N-acyl derivatives. These have been studied as growth inhibitors for microbial systems and as potential anti-tumor agents. Large scale isolation and resolution of the optical isomers are required for definitive listing, since usually only a single isomer exhibits inhibitory activity.

BIOSYNTHESIS SECTION

The name was changed from Tumor-Host Relations Section. Current studies of Drs. E. Kuff and E. Shelton in collaboration with Dr. J. T. Harrington and Mrs. K. Lueders deal with the detailed molecular structure of polyribosomes in mammalian cells.

Polyribosomes isolated from tumors following a brief (20 min.) *in vivo* incorporation of ^{3}H-uridine contain labelled RNA which is heterogeneous on sedimentation analysis and corresponds to neither the ribosomal nor transfer RNA's. This rapidly labelled RNA was assumed to represent all or part of the messenger RNA components of the polyribosomes. After polyribosomes were treated with EDTA in amounts sufficient to dissociate the component ribosomes, analysis by centrifugation on sucrose density gradients showed that the rapidly-labelled RNA was contained in structures with a wide range of sedimentation velocities; for the most part moving more rapidly than the ribosomal subunits. These EDTA-released structures, containing RNA of very high specific radioactivity relative to that of the original polyribosome fraction, were examined by electron microscopy. Both native ribosome subunits and those released from polyribosomes by EDTA had a common basic infrastructure of an amorphous background interspersed with 5-8 Å clusters staining densely with uranyl acetate which probably represent local concentrations of RNA. Sixty-S subunits released from polyribosomes exhibit 20 Å filaments, 75-100 Å in length attached to their surfaces. Such filaments are rarely seen on 40 S subunits released at the same time or on naturally occurring 45 S subunits. EDTA treatment of polyribosomes released complex branching and dividing strands, 20-50 Å wide and several thousand Å long. Distinct circular areas 175-200 Å in diameter were dispersed along the length of the complex filaments. The precise composition of these structures is not known. The tentative conclusion based on labelling data, electron microscopy, and buoyant density determinations, is that they represent complexes of messenger RNA with

protein and/or lipid material, in association with a few ribosomal subunits which have failed to dissociate from the complex upon EDTA treatment. Morphologically similar elements have been released from purified tumor cell nuclei by mild extraction procedures, without recourse to chelating agents. The nuclear-derived particles resemble those released from the cytoplasmic polyribosomes by EDTA in certain respects (content of rapidly labelled polydisperse RNA, similar buoyant density in CsCl gradients).

In the course of the above studies, a hitherto undescribed cellular entity has been discovered. The body of the particle consists of a stack of four doughnut shaped cylinders which may be independent or may have a head attached to one or both ends. The head is a single structure with a characteristic trefoil shape when attached to the body; free heads appear to have four lobes. The bodies are 100 Å wide by 150 Å long and the heads are 135 Å wide by 200 Å high. When cytoplasmic extracts are centrifuged in a 15-30% sucrose gradient; the particles sediment at approximately 32 S. They resist digestion with deoxyribonuclease, ribonuclease, phospholipase A, C and D but are sensitive to pronase. They have been found in two plasma cell tumors of the mouse, mouse liver, rabbit reticulocytes, human erythrocytes and pea seedlings but have not been found in bacteria.

Dr. E. B. Thompson, a new member of the Laboratory, is concerned at a molecular level with the mechanism of action by which steroid hormones induce enzyme formation and seeks to relate this phenomena to the regulatory mechanisms involved in mammalian cell protein synthesis. Previous work had shown that steroid hormones with glucocorticoid action induced increased tyrosine aminotransferase activity in a Morris hepatoma (7288C) cell line carried in tissue culture. It was suggested that this was a result of new protein synthesis and that continued induction required the presence of the inducing steroid.

Microanatomic studies with Drs. Edelman, Bogorach, Inman and Moses have suggested that the steroid within the cell is perinucleolar. Studies have also been started seeking to determine the effect of steroid on the intracellular sites of DNA and RNA synthesis.

Using a radioimmunological technique, it has been shown that tyrosine aminotransferase induction is due to an increased rate of enzyme synthesis, that the rate of synthesis increase precedes the accumulation of enzyme activity upon induction, and that upon removal of inducer, the synthetic rate returns toward basal levels. Experiments with Actinomycin D and other agents have suggested that the mRNA for tyrosine transaminase is relatively long-lived and that it is controlled at the translation level as well as at the level of transcription from DNA. From these experiments, a model to explain the control mechanisms involved in enzyme induction in mammalian cells has evolved; namely that there is control at two levels, at the level of gene transcription as in bacteria and also at the level of messenger RNA translation.

Dr. P. Leder and his associates (Drs. R. Erbe and D. Livingston) are seeking to define the molecular mechanisms which initiate protein synthesis, initial and subsequent peptide bond formation, which provide for the orderly translocation of messenger RNA along the ribosome during protein synthesis, and examine the steps required for the detachment and deformylation of the completed protein molecule. Rapid assay methods have been developed which permit individual detection of translation of the first and second codons of dicodon messengers, and translocation of mRNA relative to the ribosome. The use of the small messenger system and these specific assays has elucidated the function of isolated factors required for protein synthesis. Of these, at least two together with GTP, are required for the correct initiation of protein synthesis. Translation of the second codon requires the addition of a third factor, involved in a binding interaction, and is accompanied by formation of the appropriate dipeptide. Further translation of a tri-codon requires the addition of yet another factor, indicating its involvement in the translocation process. Additional studies have shown that the formyl blocking group of N-formyl-methionine-tRNA, which is required for correct, in phase initiation of protein synthesis in bacterial systems, is removed by an enzyme partially purified from E. coli. Specificity of this enzyme requires that the formyl-methionine be incorporated into peptidyl-tRNA prior to deformylation. This result is consistent with the role of the formyl group in initiation of protein synthesis and its redundancy after formation of the first peptide bond.

Dr. M. Rechcigl has been studying the alteration in the rates and in the kinetics of synthesis and degradation of catalase and related enzymes, as well as their regulation, under various physiological and pathological conditions. His numerous studies on the kinetics of catalase synthesis and destruction in vivo under various experimental conditions seem to imply that the rate of degradation, may be the preferential, although by no means the exclusive, way for the mammalian organism to control its levels of enzymatic activity in the liver. Severe starvation, dietary protein insufficiency, age, sex and species differences were all accounted for by the differential rates of synthesis. In those neoplastic tissues which have been examined to date, the modification of the rate of enzyme synthesis rather than that of its destruction accounted for the observed differences between the normal and tumor tissues. One notable exception has been two closely related C57BL substrains of mice. In this instance, an alteration in the enzyme degradation constant has been observed - a trait under a control of a single autosomal gene. Recent studies imply that the action of the gene may be indirect. Examination of catalase levels in the kidney and erythrocyte of different inbred strains of mice suggest that the enzyme activity in these tissues is controlled by different mechanisms.

In cooperation with Dr. Hruban, an excellent correlation has been evolved between the number and structure of microbodies and the activity of urate oxidase, D-amino acid oxidase, and catalase. Uricase crystals have often been seen in the microbodies by electron microscopy. Extensive biochemical and morphological examination of the opossum prostate have revealed marked localization and high concentration of catalase.

Over the past few years, Dr. P. Gullino had developed a tissue-isolated tumor system which remained connected to the host only through an afferent and efferent blood vessel. Cell free interstitial fluids could be obtained via an implanted diffusion chamber encompassed within the growing tumor. During the past year, a completely isolated system has evolved consisting of a circuit formed by two pumps, a pulsatile "minilung" system, a microchamber for continuous pH determinations, a dialyzing system and 2 containers, one for the tumor and the other for the perfusate. Three perfusates were used, heparinized total blood, blood diluted with Eagle's medium 2:1 and an erythrocyte-free perfusate formed by Eagle's medium + rat serum (20%) + 7 gm % of bovine albumin. With this system, it was found that the flow-pressure curve of the tumors perfused with total or diluted blood had a shape peculiar to neoplastic tissue characterized by a sharp increase in resistance to flow, regardless of the flow rate. This initial hypertensive phase is unexplained. The second part of the curve is characterized by a disassociation of flow and pressure. Within certain limits, it is possible to double the flow with very little increase in pressure. The third part of the curve manifests itself in a hypertensive crisis followed by irregular changes in flow until perfusate can no longer flow through the tumor despite a very high pressure. Perfusates without erythrocytes do not present this last hypertensive phase and perfusion can be continued for several days.

Walker carcinomas perfused with total blood transformed about 50% of the utilized glucose into lactate. This was higher than the *in vivo* value (35%). If blood was diluted or erythrocyte-free perfusate was used, about 80% of the utilized glucose was transformed into lactate. There was practically no difference in lactate production whether perfusates with or without erythrocytes were used, despite a 10-fold reduction in O_2 utilization observed with erythrocyte-free perfusates. Long term perfusions (more than 24 hours) with erythrocyte-free perfusate were unsuccessful. However, Walker carcinomas perfused for 50-60 hours with perfusates having a 7% hematocrit (about 2 gm Hb %) survived and were transplantable.

The ability of mammary carcinomas to remove O_2 from the afferent blood was as good in perfused tumors as *in vivo* provided that total blood was used. Dilution of blood *per se* impaired the oxygen removal capacity of the tumor. For the tumor, the erythrocytes represent an O_2 supply released during passage through the neoplastic vessels. Lacking this reservoir, the tumor was unable to lower the O_2 content of the fluid phase of the perfusate below a pO_2 of 90 mm Hg, and the limiting factor was the amount of

fluid which could be passed through the tumor. The ability of mammary carcinomas to remove glucose from the afferent blood was equal to or larger than in vivo. The total consumption, however, was smaller than that in vivo because the maximal amount of blood which could be perfused in vitro was smaller than the flow measured in vivo. A surprising dissociation between O_2 and glucose consumption was observed.

In experiments designed to study factors which would influence the in vivo transfer of substances into neoplastic cells, Drs. Fiszer-Szafarz and Gullino found that the hyaluronic acid (HA) content of the interstitial fluid of Walker carcinomas was about 1/2 that found in the subcutaneous area where the tumor was transplanted. Chondroitin sulfuric acid was present in the subcutaneous interstitial fluid (SIF) but not in tumor interstitial fluid (TIF). The degree of polymerization of HA in TIF was less than in SIF. The presence of the tumor reduced to about 1/2 the HA concentration in the SIF of the tumor-bearing host. The hyaluronidase activity of TIF was about 1/3 higher than that of the SIF despite a decrease below normal of hyaluronidase activity in the serum of the host. These findings are a first step toward a more complete analysis of the conditions which may influence the "permeability" of the vascular and interstitial spaces of mammary tumors.

The uptake of lactating mammary gland DNA by non-functioning mammary tumors is being examined by Drs. C. Watters and Gullino, who found that DNA added to whole or diluted blood can be perfused through a mammary tumor for 5 to 6 hours without any major change in the molecular weight profile of the DNA. The amount of DNA taken up by erythrocytes during perfusion was negligible while the uptake by the Walker carcinoma itself was in the order of 0.3% of the total DNA content of the tumor. These experiments represent initial phases of a study of the biological effects of DNA extracted from functional tissue and introduced into non-functioning tissue.

NUCLEIC ACIDS SECTION

The objectives of Dr. W. Schneider's research are to isolate and identify the deoxyribose-containing compounds present in normal and cancer tissues and to determine the metabolic sequences in which they are involved and their relationship to DNA synthesis. Normal rat liver, regenerating rat liver and the Novikoff hepatoma are the main tissues used in these studies.

Research work during the past year dealt entirely with the occurrence of small amounts of DNA in a new subcellular component, the microsomes or endoplasmic reticulum. It has been found that the incorporation of thymidine into microsomal DNA was intermediate in extent between that of nuclear and mitochondrial DNA in both rat and mouse liver. The possible mitochondrial contamination of the microsome fraction could readily be measured using a marker enzyme and was demonstrated to be less than

2 per cent. Calculation of the microsomal DNA specific activity from this value showed it to be much lower than the observed specific activity. Another possibility considered was that the microsomal DNA represented a high specific activity nuclear DNA that leaked out during the preparation of the microsomes. Although it was possible to demonstrate leakage of DNA from liver nuclei, the specific activity of such DNA was considerably lower than that of the microsomal DNA. The latter also differed from nuclear DNA in its difficulty of extraction and insensitivity to deoxyribonuclease. While in these respects, the microsomal DNA resembled mitochondrial DNA, examination of DNA purified from microsomes, however, showed that it resembled nuclear DNA in every other respect. Both DNA samples had the same buoyant density in CsCl, sedimentation rate and melting temperature. Microsomal and nuclear DNA were both linear in form by electron microscopy and both contained satellite DNA bands when isolated from mouse liver. The functional significance of microsomal DNA tested for via RNA polymerase activity , showed that microsomes possessed this activity to about the same degree as nuclei, but a requirement for DNA in the reaction could not be demonstrated with microsomes, perhaps explained by the relative inaccessibility of microsomal DNA which has already been noted. Localization studies indicated that the DNA was associated with the membranous portions of the microsomes.

Dr. Stephen Lerner has been examining the extent of complementarity between mitochondrial RNA and DNA with the aiming of understanding the biosynthetic processes of mitochondria and its control. To this end, procedures for preparing DNA and RNA from mitochondria free from cross-contamination are necessary. Separation of the various species of RNA was carried out on sucrose density gradients with fair to good resolution of peaks. Major peaks were found at approximately 4, 8, and 28 S from nuclear, mitochondrial, and cytoplasmic preparations. Mitochondrial preparations had variable amounts of minor peaks, one in the valley between 4 and 18 S peaks, and two on the heavy side of the 18 S peak. Ribonuclease activity present in the preparations was estimated from sucrose gradient patterns before and after incubation. Degradation of RNA could be recognized by diminution of 18 and 28 S peaks and a concomitant rise in the 4 S and minor peaks. Mitochondrial RNA preparations have finally been obtained which exhibited little alteration of peaks after incubation at 25°C for 24 hours. Labeling of RNA's was accomplished by intraperitoneal injection of 3 mc of 5-^{3}H-orotic acid in divided doses over 24 hours into each rat. Specific activities on the order of 1 million cpm per micromole of RNA-phosphorous were obtained.

Dr. W. G. Fiscus is engaged in the purification and characterization of phosphorylcholine-cytidyl transferase. Interest in this enzyme stems from observations previously obtained in this laboratory that a product, dCDP-choline is present in tumor tissue in much higher concentration than in normal liver and that the enzyme is activated by altered phospholipids in a

manner suggesting a regulatory role in phospholipid metabolism. Partial purification of the enzyme has been accomplished resulting in 45-fold increase in specific activity with good recovery of total activity. However, it has not yet been possible to either obtain high levels of purification or to study the properties of the partly purified enzyme due to instability of activity. An inhibitor of this enzyme, possibly a polyribonucleotide, has been found in rat liver.

Dr. R. Kielley's work on the purification and characterization of thymidylate kinase (dTMP kinase) is part of an attempt to understand the activation and repression of this key enzyme in the regulation of DNA synthesis. Previous work had shown that a large part of the cellular dTMP kinase of mouse liver was held in an inactive state from which it could be released with resultant activities equal to that of hepatoma. Enzyme purification methods included the usual techniques of extraction, acid preparation, ion-exchange chromatography, salt precipitation and gel filtration which were used with the object of obtaining maximum enzyme purity with the highest efficiency. These considerations were of more than usual significance because the source material, mouse ascites hepatoma, does not permit accumulation of large amounts of material; the amount of dTMP kinase to be expected in any tissue is extremely small; and the necessity to keep the enzyme protected by its substrate, dTMP, throughout purification. The highly purified dTMP kinase finally obtained represents about a 7000-fold purification over the enzyme in the crude extract, with an overall recovery of about 15%. The amount of enzyme recovered from 500 g of cells was less than 1 mg. In the final gel filtration step, the enzyme corresponded to a distinct protein peak, but showed some overlap with adjacent protein components. Preliminary studies on the purified enzyme indicate that it is strictly a thymidine monophosphate kinase containing less than 0.5% of diphosphokinase. No kinase activity against other nucleoside monophosphates tested could be detected by means of the coupled spectrophotometric assay for ADP. Adenylate kinase was the chief contaminant and the last traces were eliminated only in the final step.

Dr. J. Rotherham is interested in the nature and extent of the biochemical reactions by which nucleic acid precursors are metabolized in cultured nonmalignant and neoplastic cells, particularly with respect to their possible significance in nucleic acid synthesis and neoplastic transformation. A neoplastic murine cell strain, NCTC 3681, grown in chemically-defined medium for several years, utilized labeled thymidine or deoxycytidine for DNA-thymidine synthesis to a greater extent than it did cytidine, uridine, deoxyuridine or orotic acid. When the folate content of the medium was increased ten-fold, there was a greater utilization of cytidine for both DNA pyrimidines and, to a lesser extent, an increase in the utilization of deoxycytidine, deoxyuridine and uridine for DNA-thymidine. A cell strain derived from strain NCTC 3681, by growing these cells for 13 transplant generations in medium containing the ten-fold higher level of folate, showed a marked increase in the extent of utilization of cytidine,

uridine and deoxyuridine for DNA-thymidine by the derived cells and the specific activity ratios of DNA-deoxycytidine to DNA-thymidine decreased. The extent of utilization of orotic acid remained comparatively low. A study was made of the incorporation of precursors into the nucleic acids of NCTC 4823 cells, a non-neoplastic murine cell strain using similar methods. The data are incomplete for the 4823 cells but some apparent differences were seen in the extent of utilization of some of the precursors by the two cell strains.

Investigations have been undertaken by Dr. E. P. Anderson to delineate the relationship between biological sensitivity or resistance to fluorouracil and to azauracil in mouse tumor sublines, and the levels of uridine kinase in these lines.

Current work is concerned with the salvage pathways of pyrimidine nucleotide biosynthesis, their enzymology, and mechanisms by which they may be controlled. Studies on resistance to pyrimidine analogs have revealed three resistant tumor lines (two fluorouracil-resistant lines of the P815 mast cell tumor and one azauracil-resistant line of the 70429 plasma cell neoplasm) that are deficient in uridine kinase, a primary enzyme in the salvage pathway for anabolism of uracil and its analogs. The kinase from sensitive and resistant cells has been found to be similar in many of its properties; it is now being purified by Mr. J. Ciardi in parallel from both cell lines for more detailed studies. Uridine kinase has been shown to be the rate-limiting step in uridine anabolism and has been found to be feedback controlled by the end-products, UTP and CTP. In Tetrahymena pyriformis, in which this is not the primary salvage pathway for uracil anabolism, this enzyme was found to be insensitive to such feedback inhibition. In order to explore possible mechanisms of control over subsequent steps in the anabolic pathway, Dr. Ruffner has purified UMP kinase 300-fold from Tetrahymena and is studying its inhibition by nucleotides. Dr. Lyon studied thymidine-deoxyuridine phosphorylase (an earlier step in uracil metabolism), which he purified 60-fold from Tetrahymena; his data indicated that this enzyme binds the pyrimidine in two distinct ways, one at the catalytic site, and a second at a non-catalytic site in a manner inhibitory to the reaction in either direction; the two sites can be distinguished in the presence of urea which presumably affects the conformation of the protein. This could also serve as a mechanism to control the activity of this enzyme.

Dr. G. Rushizky's studies of the structure and function of nucleic acids deal with the RNA of E. coli phage MS2 and the DNA of calf-thymus. Methodology has been developed to obtain oligomers of 10-250 nucleotides in length by nuclease digestion of nucleic acids attached to DEAE-cellulose and DEAE-Sephadex. Oligomers of DNA obtained in this manner corresponded in average chain length to fractions analogously prepared from RNA. Base ratio variations between the oligomer fractions and the original DNA were also found. However, these base ratio variations indicate a preferential binding of purine-oligomers to the adsorbent DEAE-cellulose, rather

than a restricted specificity of enzymatic action. Oligomers so obtained have been used for computer assisted spectral analysis. Prior to an investigation of divalent metal catalyzed, partial degradation of RNA, the metal content of RNA and DNA has been examined by neutron activation as a function of previous treatment of the RNA, in collaboration with Dr. W. W. Miller of Pennsylvania State. Preliminary results show that CM-cellulose treated RNA is largely free (less than 2 ppm of Na) of Na^+ and K^+, which would interfere with metal analysis because of their high energy gamma radiation. Neutron activation should give more accurate results than atomic adsorption methods, since for the latter the presence of phosphorus requires muffle furnace temperatures of 700° or more which may volatilize some metals.

Polynucleotide kinase can be used to label nucleotide chains by virtue of its ability to transfer ^{32}P from ATP^{32} to the 5'OH termini of oligomers. In our hands, however, this enzyme is contaminated with RNase when prepared as described by others, even when isolated from the A-19 "RNase-free" strain of E. coli. Furthermore, more than 50-fold molar excess of ATP is needed to label an oligomer. Further purification of the enzyme and kinetics of RNA end group labeling are under investigation.

In a continuing study of the nature of the forces and the specificity of interaction between proteins and nucleic acids, Drs. Latt and Sober reported that equilibrium dialysis measurements were made at pH 7 and 4° over a range of NaCl concentrations of the binding of individual oligomers of the (L-lysine)$_n$-ε-N-DNP-L-lysine series (n = 3, 4, 5, 6, 7 or 8) to synthetic polynucleotides, principally poly (I + C) and poly (A + U). Evidence was presented for a 1:1 lysine:phosphorus ratio in the soluble complexes formed. Binding was stronger to poly (I + C) than to poly (A + U). Both the total binding energy and the difference between the binding energies to poly (I + C) and poly (A + U) increased linearly with oligolysine chain length. The strong inhibition of the binding by NaCl is interpreted in terms of a competition between Na^+ and the oligolysines for the polynucleotide phosphates. A general theory of reversible colinear oligomer-polymer interactions was presented and used to extract parameters from the binding data.

Cations were shown to affect the extent and specificity of binding of individual members of the series (L-Lys)$_n$-ε-N-DNP-L-lysine (n = 3, 4, 5, 6, or 7) to synthetic polynucleotides, poly (A + U) or poly (I + C). Interpreting this effect as the result of competition between the cation and the oligolysine for the polynucleotide phosphates, it was possible to group cations into those with a binding preference for poly (A + U) [$(CH_3)_4N^+ > Li^+ > Na^+$; Mg^{2+}, Ca^{2+}, Mn^{2+}]; those with little specificity for either polynucleotide [Lys-H^+, K^+, TrisH$^+$, NH_4^+]; and those with a binding preference for poly (I + C) [ArgH$^+$, HisH$^+$]. Cation specificity for poly (A + U) correlated with cation ability to organize water structure and with volume increase on

cation neutralization of polyphosphates, suggesting that specificity in protein-nucleic acid interactions might be directed by the solvent structure surrounding the interacting species. This grouping according to solvent structure promotion also held regarding cation effect on messenger stimulated binding of tRNA to ribosomes, K^+, $TrisH^+$, and NH_4^+ promoting this binding and $(CH_3)_4N^+$, Li^+, and Na^+ inhibiting it, suggesting a connection between some specific cation effects in biological systems and cation binding to phosphate.

Chromosomes represent a biologically significant fractionation of nucleoproteins. In an investigation concerned with the structure and interactions of proteins and nucleic acids in a native state, Drs. O. W. McBride and H. A. Sober are attempting to study chromosomes isolated from mitotic cells. In order to obtain large numbers of cells in metaphase, the technique of single and double block with excess thymidine has been used to obtain a limited, but useful, degree of synchronization of cell growth. Other methods utilizing reduced temperature of cold shock were found to be much less successful. Several methods of chromosome isolation have been compared. "Metaphase plates" and nuclei isolated in hexylene glycol and 1-2.5 x 10^{-4}M $CaCl_2$ contain significant amounts of adherent cytoplasm. The cytoplasmic contamination can be prevented by decreasing the calcium concentration further and increasing the shearing force but concurrently there occurs deterioration of the morphology of the chromosomes. The complete removal of cytoplasm from chromosomes isolated in non-ionic detergents is found only in the presence of very low concentrations of divalent cations and is associated with deterioration of the chromosome morphology. Two recent methods proposed by Maio and Schildkraut and by Huberman and Attardi have been the most useful though some cytoplasmic contamination of chromosomes is present in these procedures also.

PROTEIN CHEMISTRY SECTION

Methods for the fractionation of blood and bone marrow cells developed by Drs. E. Peterson and W. Evans, have been applied to a number of specific problems. Work by Drs. Evans, Brubaker, Mage and Peterson is directed toward the separation of leukocytes according to their stage of maturation. Erythrocytes, lymphocytes, monocytes and granulocytes from normal human blood have been separated using the previously developed 1 x g sedimentation method. Peak tubes of the various fractions showed the following purities: erythrocytes 96%, lymphocytes 75%, monocytes 93%, granulocytes 98%. Sedimentation of blood cells from a patient with chronic myelogenous leukemia resulted in a complete separation of erythroid from myeloid cells. The more immature myeloid cells (i.e., myelocytes, promyelocytes) sedimented more rapidly than mature myeloid cells (i.e., segmented and band granulocytes) yielding purities of up to 88% for the former and 90% for the latter). A guinea pig bone marrow cell preparation enriched in

myeloblasts and immature granulocytes has been obtained, using a modification of the glass bead column cell separation method. Bone marrow cells are passed through a horizontal rotating column filled with serum-coated glass beads at room temperature. Up to 90% of the mature granulocytes adhere to such columns whereas the more immature granulocytes and blast cells pass through unadsorbed and are collected in the effluent. Most of the red cell precursors (60%) and monocytes (100%) are also removed by this process, which requires about 60 minutes to complete.

Studies are in progress on the factors which regulate enzyme formation and activation during the maturation of bone marrow granulocytes. Myeloperoxidase has been chosen as a model enzyme for these studies and fractionations have been obtained which provide highly purified myeloperoxidase from the bone marrow cells of the guinea pig. Multiple components of this enzyme or additional enzymes of related specificity have been found. The purified protein will be used to produce an immunological reagent for studying the interrelationships of these components and the time course of assembly of the enzymes during differentiation.

The purification and characterization of immunocytes is being continued by Drs. M. Mage, W. Evans and E. Peterson. They have found that on sedimentation at 1xg in a sucrose gradient, most of the IgM and IgG antibody plaque-forming cells (PFC's) sediment more rapidly than do most of the non-PFC's. Thus, antibody PFC's have been enriched up to 18-fold over the unfractionated suspension. Passage of spleen cell suspensions through antigen (sheep erythrocyte)-coated polyurethane foams results in the binding of most of the PFC's,which can subsequently be released by mechanical agitation and assayed. When spleen cells from a mouse immunized with sheep erythrocytes (SRBC's) were passed through such an antigen-coated foam, only 15% of all nucleated cells were bound, but these included 60 to 90% of the PFC's. With unwashed spleen cells,27% of all nucleated cells were bound, including 70% to 80% of PFC's. When spleen cells from a mouse immunized with SRBC's were passed through a foam coated with human erythrocytes, only 6% of total nucleated cells and 6% of PFC's were bound. Nucleated cells from unimmunized mice were bound to the extent of 8-10%. The difference in binding of anti-SRBC spleen cells to SRBC-coated foam (15% of total nucleated cells) compared to their binding to a human erythrocyte coated foam (6%) indicates that approximately 9% of the total nucleated cell load of 1.5×10^7 nucleated cells were specifically bound to the SRBC-coated foam, over and above background. Although from 60 to 90% of the PFC's were bound, these constituted less than 1% of the bound nucleated cells. The other 99% of the specifically bound nucleated cells may be producers of non-hemolytic anti-SRBC antibodies, or may be cells that are passively coated with anti-SRBC antibody, causing them to bind specifically to SRBC's.

The studies on cell binding to antibody-coated foams are being extended to glass bead columns as a matrix for separation. Here, the beads were first coated with rabbit IgG, then with specifically purified anti-rabbit IgG, which in turn bound rabbit antibody that was specific for sheep erythrocytes. The capacity and efficiency of binding of SRBC's to such columns was higher than for antibody-coated foam columns. However, when nucleated cells were passed through such glass bead columns, high non-specific binding remained a problem.

Studies of the homogeneity and structure of proteins have continued. Dr. S. Ralph Himmelhoch has simplified and routinized the isolation of leucine aminopeptidase from swine kidney. The product has been examined extensively by techniques developed to detect endopeptidase contamination and has been found to be free of such activity, thus making this enzyme suitable for use in peptide and protein sequence analysis. The relationship of metals to the activity of the enzyme has been reexamined. The enzyme contains, by analysis, from 2.6 to 4 gram atoms of zinc/mole of protein (300,000 g). It is inhibited by a wide array of chelating agents with an affinity for Group IIb metals. The zinc can be removed from the enzyme with concomittant loss of activity or replaced by cadmium to give an inactive form of the enzyme, which can be reactivated by replacement of cadmium by zinc. The role of Mn activation of the enzyme has been found to result from the replacement of a portion of the native metal (zinc) by Mn^{2+} under the conditions of the assay.

Dr. O. W. McBride has been studying the insoluble vertebrate tropocollagens that can be solubilized at neutral pH by mercaptoethylamine. Another thiol reagent, dithiothreitol, was also found to be effective, whereas the chelating agents, EDTA and o-phenanthroline, as well as a general reducing agent, sodium borohydride, were ineffective at pH 7.4. Rat skin collagen oxidized by performic acid contains about 0.5 residue of cysteic acid per 1000 residues of total amino acids. The apparent cysteic acid content of this collagen was not altered when the denatured protein in 5M guanidine hydrochloride was passed through Sephadex G-200 to remove any low molecular weight contaminants. α_1 and α_2 chains isolated by CM cellulose chromatography contained only 0.2-0.3 residue of cysteic acid per 1000 residues of amino acid, whereas the dimers, β_{11} and β_{12}, have cysteic acid contents of about 1.0 and 0.3 residue per 1000 residues of amino acid, respectively. The diagonal electrophoresis method of Brown and Hartley has been employed to separate the soluble peptides produced by the digestion of collagen by collagenase. A single, off-diagonal peptide was found after performic acid oxidation. Its high content of glycine, proline, and hydroxyproline indicate that it was probably derived from collagen, rather than from a contaminant, but it has not yet been demonstrated to contain cysteic acid.

Dr. R. Hartley together with Dr. E. A. Barker and J. R. Smeaton have been engaged in the purification, characterization and investigation of an extracellular ribonuclease of B. amyloliquefaciens (formerly considered a strain of B. subtilis) and its natural inhibitor (from the same source) and in the development of methods of culture and genetic analysis so that the organism and its product pair may be applied to a long-range study of enzyme structure, function, and synthesis.

The extracellular enzyme has been found to undergo a reversible thermal transition that synchronously affects ultraviolet absorption, optical rotation, and gel filtration behavior. It has been concluded that the transition is highly cooperative and involves extensive disorganization of the native structure. The transition temperature and the ΔH of the transition are affected by urea, by extremes of pH, and in different ways by cupric and sulfate ions. They are not affected by phosphate or substrate analogs. A fully succinylated derivative is inactive but maintains essentially the native conformation, with reduced stability. At least three of the 6 or 7 tyrosines and at least one of the tryptophans are buried in the folded form and inaccessible to solvent. The molecular weight of the enzyme has been redetermined as 12,100. Crystals of the enzyme may be obtained in either high or low salt.

The development of micro methods for "dansyl", Edman, and gas-liquid chromatographic techniques has made practical the use of peptides isolated from paper maps and has accelerated the determination of the amino acid sequence. Several short sequences are now known and the amino acid compositions of several tryptic peptides obtained. The N-terminal (alanine) was previously reported, and the C-terminal (arginine) has now been established.

The inhibitor which is an intracellular protein, has been obtained in relatively pure form, as judged by disc electrophoresis and by experiments involving complexing with the enzyme. Inhibition involves the formation of a one-to-one complex between enzyme and inhibitor. The reaction is reversible in urea solution and both activities may be recovered from the complex.

A B. subtilis phage (SP-10) has been adapted to grow in some of our ribonuclease-producing strains, but others appear to be resistant. Preliminary efforts to transduce nutritional factors have not been successful.

Dr. E. Peterson, with the cooperation of Linda Fryklund (Institute of Biochemistry, Uppsala, Sweden), has used polyamino acids to study the applicability of ion exchange electrophoresis to the separation of polyelectrolytes of higher molecular weight. Chromatographic tests have demonstrated that the amphoteric adsorbent made with glycine can be used to fractionate polylysine oligomers at pH 5.3, employing a gradient of pyridine acetate for elution, instead of the LiCl or NaCl required for elution from CM cellulose. Since the pyridine acetate can be evaporated

readily, the effluent fractions are easily freed of salt. By adjustment of the pH it was possible to alter the effective number of binding groups on the adsorbent to suit the requirements for light binding of the larger oligomers of polylysine, permitting their migration in a strong electric field. However, conditions have not yet been found that permit simultaneous electrophoretic resolution of polylysine oligomers over a wide range of oligomers, although the range attained is significantly wider than that obtained with CM cellulose. A column was constructed for high voltage experiments on a larger scale, incorporating means for removal of the migrating bands without interrupting electrophoresis. Separation of oligomers of DNP-polylysine (yellow in color) could be clearly seen and the bands could be collected, but factors inherent in the method, itself, caused excessive dilution during collection.

Lysine oligomers continue to serve as model substances for the study of basic proteins by Dr. H. A. Sober and members of the Weizmann Institute of Science under collaborative agreement no. 235103. Oligomers of known chain length, composition and sequence have been prepared and the separation processes have been modified so that individual oligomers of chain lengths as high as 30 can be prepared. They have been used in the following studies: a) the effect of chain length on adsorption to ion exchangers and behavior on Sephadex and polyacrylamide gel-filtration columns, b) optical rotatory dispersion studies have been completed. Mathematical computations have been used for analysis of the data and a manuscript is being prepared for publication, c) interaction with other polyelectrolytes, primarily polynucleotides and nucleic acids, d) the Merrifield procedure has been used to prepare oligomers of lysine, histidine and ornithine. Chromatographic analysis of the products has indicated that premature termination and cross-linking of chains occur, providing unwanted and spurious products.

Experiments performed by Dr. Michael Feldman at the Weizmann Institute of Science under a collaborative agreement with Dr. Sober (P. L. 480 no. 335105) continue to examine the mechanisms of immunological induction and tolerance. Results obtained to date imply that the regeneration of immune competence following exposure to total body irradiation depends not only on the regeneration of lymphocytes, but also on the post-radiation recovery of another cell type which may be essential for triggering antibody production. An obvious candidate is the macrophage. In vivo studies have shown that the immune response could not be attributed to lymphoid elements in the macrophage cell population but that "primed" macrophages appear to act upon recipient lymphocytes to induce the immune reaction. In vitro experiments on the induction of antibodies, carried out in the organ culture system of Globerson and Auerbach in the presence of irradiated spleen and thymus explants, led to the same conclusions.

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Other:	4

Project Description:

Objectives: The objectives of the Administrative Unit in the Office of the Chief are to maintain personnel, correspondence, reprint and requisition files, maintain leave, overtime and budget records, maintain a library, arrange for travel for professional staff, compose and type letters, memoranda, requisitions, shop orders, type manuscripts, lantern slides, travel requests, personnel requests and miscellaneous charts and articles. The above duties are performed for 42 professional employees.

Methods: Since July 1967, this unit has typed the following: Approximately 3050 pages of original manuscript, 325 letters and memoranda, 10 lantern slides, 400 pages of miscellaneous material, 1542 requisitions (531 TCO's) 175 instrument shop orders, 16 requests for services, 14 shipping requests, initiated 65 travel orders, issued 63 TR's, completed 63 vouchers, (includes 1 foreign travel order) and 31 personnel action requests.

170 calls to Systems Maintenance Unit concerning care for all types of laboratory equipment. Follow up on these calls. Initiated 80 work requests concerning maintenance, fabrication and repair to laboratory spaces and equipment. Acted as liason between investigators and shop sections in all of the above. Maintained logs and records of same.

Approximately 250 calls to building maintenance unit with the necessary follow up. Supervised complete renovation of 3 Laboratory spaces and

coordinated work with shop areas. Initiated original and reviewed all other plans submitted by investigators for approximately 60 laboratory spaces in building 37. Acted as principal contact between plant engineering personnel and investigators for planning purposes. Acquired data and technical information on new equipment to be used in new laboratories. Acquired, evaluated, maintained and stored equipment that will be utilized in new facility.

Orders were placed with animal production unit for 9,220 mice, 10,146 rats, including 595 germ free, 172 guinea pigs, 40 rabbits and 2 hamsters. Records were maintained as to date of receipt, strain, sex, age, weight, and health condition.

Supervised and maintained Xerox copy machine - output during the past year. Acquired and maintained supplies for above.

Inventory and records maintained on laboratory equipment including new receipts, receipts from and transferred to property utilization. Served as property custodian for laboratory.

Daily visits to laboratories with personal contact with investigators as to possible need of repair, maintenance or technical or technical problems, etc. Daily check on all animal rooms to insure proper care of laboratory animals.

Approximately 55 visits to property utilization (SMB-surplus), to try to locate usable equipment for the Laboratory of Biochemistry.

Stocked and maintained chemical storage area including gas cylinders, and liquid solvents.

Honors and Awards: None

Publications: None

Serial No. NCI-302
1. Laboratory of Biochemistry
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Employment of microchemical methods for the analysis of biological samples arising from a basic examination of the neoplastic processes. Routine microchemical elementary analysis. Routine amino acid and protein analysis using the spinco model 120 amino acid analyzer and modification thereof.

Principal Investigator: James F. Williams

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	0
Other:	1

Project Description:

Objectives: The microchemical unit provides a complementary analytical service functioning in direct collaboration with the independent investigators of the Laboratory of Biochemistry.

The routine service of the unit continues as described in previous annual reports. During the period 2900 biochemical samples were analyzed, primarily for nitrogen. A total of 325 amino acid, chromatographic analyses were performed. These were primarily ninhydrin colorimetric analysis of oligomers of amino acids with the aid of ion exchange cellulose columns to identify specific oligomers and to assess their purity. There were also colorimetric analyses of protein hydrolysates performed on Dowex resins. The Amino Acid Analyzer also required routine maintenance to be performed as needed.

Honors and Awards: None

Publications: None

Serial No. NCI-339
1. Laboratory of Biochemistry
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Oligonucleotides: properties and biological activity.

Previous Serial Number: Same

Principal Investigator: Herbert A. Sober

Other Investigators: Michael Feldman

Cooperating Units: Laboratory of Experimental Biology, Weizmann Institute of Science, Rehovoth, Israel with Dr. Michael Feldman under Public Law 480 Agreement no. 335105

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives: To study pure oligonucleotides of known chain length and nucleotide sequences in terms of their chemical, physical and biological properties.

Methods: RNA , DNA, and nucleotide polymers are partially and completely digested by specific and non-specific nucleases and the hydrolysates are fractionated by chromatography on DEAE-cellulose, Sephadex, polyacrylamide gels, as well as by paper electrophoresis and chromatography. Oligonucleotides are examined by physico-chemical techniques (e. g., ultraviolet spectra, optical rotatory dispersion, etc.) and tested for activity in stimulating plasma cell differentiation and in reactivating the immune response in tolerant animals.

Results: Oligomers prepared have been used in studies of nuclease specificity, neighbor-neighbor interactions in a single oligonucleotide strand and as test materials for computational analysis of nucleic acid spectra.

Experiments performed by Dr. Feldman at the Weizmann Institute of Science under the collaborative agreement continued to examine the mechanisms of immunological induction and tolerance. Studies on the kinetics of induction of immunological tolerance to human serum albumin (HSA) in adult rabbits exposed to sublethal doses (550 r) of total body x-irradiation, have revealed that tolerance can be induced with small amounts of antigen, administered after irradiation, which in non-irradiated animals will constitute small immunizing doses. The tolerant response could not be attributed to the quantitative ratio of antigen to target cell, nor can it be reconciled with the notion that tolerance induction depends on the interaction of antigen with "immature" lymphoid cells. Smaller amounts of antigen are required for the induction of tolerance when antigen treatment is started 4 weeks after irradiation than when the same treatment is initiated after 24 hours only. Since at this post-irradiation stage the lymphoid regeneration has reached normal levels, it appears that the lymphoid system recovers from radiation injury independently and in advance of the return of immunocompetence.

Thus, the regeneration of immune competence following exposure to total body irradiation seems to depend not only on the regeneration of lymphocytes, but also on the post-radiation recovery of another cell type which may be essential for triggering antibody production. An obvious candidate is the macrophage.

In vivo studies of the immunogenic function of macrophages have shown that the immune response could not be attributed to the lymphoid elements within the macrophage cell population since lymphocytes of either lymph node or thoracid duct origin, when incubated with the antigen, did not elicit antibody production in x-irradiated recipients. Pure populations of macrophages on the other hand, obtained following in vitro culturing of peritoneal cells, elicited antibody production in sublethally irradiated mice following interaction with Shigella antigen. The macrophages appear to have acted upon the recipient lymphocytes, since "primed" macrophages did not trigger antibody production in mice whose lymphoid cells were severely depleted by high doses of total body irradiation. Such depleted animals could, however, be immunologically reconstituted if inoculated with "primed" macrophages and non-treated lymphocytes from either lymph nodes or thoracic duct. The inoculation of each of these components alone did not evoke

an immune response. Thus, the interaction between two cell types in the immune response to Shigella antigen has been demonstrated and the macrophages were shown to be capable of triggering antibody production across H-2 barriers.

In order to further analyze the cellular basis of the immunological reactivation of x-irradiated animals and of the macrophage-lymphocyte interaction, experiments on the in vitro induction of antibodies were carried out by the organ culture system of Globerson and Auerbach. The system was adapted to the production of a primary immune response to Shigella antigen, by cultures of spleen explants from normal non-irradiated donors. Agglutinins to Shigella produced in vitro could be detected and measured both in the culture medium and adsorbed to the millipore filter. With this system, it was possible to test whether the production of antibodies by x-irradiated mice which had been treated with "primed" macrophages, was carried out by lymphocytes which had survived the depletion by x-rays, or by lymphocytes which had differentiated after irradiation. Macrophages which had interacted in vitro with Shigella were seeded into cultures of spleen explants obtained from animals which had been exposed to 550 r. No antibodies were produced. When, however, the macrophages were added to cultures containing both explants of irradiated spleens and thymus - agglutinins to Shigella were formed. We demonstrated previously that postirradiation regeneration of spleen lymphocytes is thymus-dependent. It therefore appears from our in vitro experiments that when macrophages activate agglutinin production in x-rayed animals, they signal lymphocytes which have been produced after exposure to x-rays, due to the inductive effect of the thymus.

Honors and Awards: None

Publications: Work performed at the Weizmann Institute of Science, Rehovoth, Israel under Public Law 480, Agreement No. 335105.

1. Feldman, M. and Gallily, R. : Cell interactions in the induction of antibody formation. Proc. Cold Spring Harbor Symp. on Antibody Production, New York, 1967.

2. Feldman, M. and Gallily, R. : The function of macrophages in the induction of antibody production in x-irradiated animals. In: Ontogeny of Immunity, Smith, R. T. (Ed.), Gainesville, Univ. Florida Press, 39 (1967).

3. Gallily, R. and Feldman, M. : The cellular components in the induction of antibody by x-irradiated animal. In: Germinal Centers in Immune Responses, Cottier, H. (Ed.) Berlin, Springer, 333 (1967).

4. Nachtigal, D. Lymphoid regeneration following x-ray treatment and the susceptibility to the induction of immunological tolerance. In: Germinal Centers in Immune Responses. Cottier, H., (Ed.) Berlin, Springer, 329 (1967).

5. Feldman, M. : The immunogenic function of macrophages. Proc. of VIIth International Society for Cell Biology Symposium on "Differentiation and Immunology," Gatlinburg, Tennessee, Oct. 1967 (in press).

Serial No. NCI-5201
1. Laboratory of Biochemistry
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Protein-Nucleic Acid Interaction.

Previous Serial Number: Same

Principal Investigator: Herbert A. Sober

Other Investigators: Samuel A. Latt

Cooperating Units: Peter Bent Brigham, Boston, Massachusetts

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives: To study the nature of the forces and the specificity of interaction between nucleic acids or polyribonucleotides and oligolysines as a model of nucleic acid-basic protein structure and function.

Methods: A series of ε-DNP-oligolysines (ε-DNP-Lys(Lys)$_n$ was prepared with chain lengths (n) ranging from 3 to 8 by fractionation on carboxymethyl cellulose of a crude mixture obtained from ε-DNP-lysine initiated polymerization (n = 13). Oligolysine chain length calculated from total lysine content and DNP absorption at 360 mμ agreed with assignments made on the basis of chromatographic position. The binding of these labeled oligomers of lysine to various RNA's and polyribonucleotides was measured using equilibrium dialysis. Polynucleotide concentrations and extinction coefficients were determined by UV absorption and total phosphorus measured; those of ε-DNP-oligolysines by 360 mμ absorption based on dry weight.

The ε-DNP-Lys(Lys)$_n$ was also used as an indicator of the binding of other (unlabeled) molecules by measuring the competition of these unlabeled molecules with the labeled ones for the binding to polynucleotide.

Major Findings: Equilibrium dialysis measurements were made at pH 7 and 4° over a range of NaCl concentrations of the binding of individual oligomers of the $(L\text{-lysine})_n$-ε-N-DNP-L-lysine series (n = 3, 4, 5, 6, 7 or 8) to synthetic polynucleotides, principally poly (I + C) and poly (A + U). Evidence was presented for a 1:1 lysine:phosphorus ratio in the soluble complexes formed. Binding was stronger to poly (I + C) than to poly (A + U). Both the total binding energy and the difference between the binding energies to poly (I + C) and poly (A + U) increased linearly with oligolysine chain length.

The strong inhibition of the binding by NaCl is interpreted in terms of a competition between Na^+ and the oligolysines for the polynucleotide phosphates. A general theory of reversible colinear oligomer-polymer interactions was presented and used to extract parameters from the binding data.

Cations were shown to affect the extent and specificity of binding of individual members of the series $(L\text{-Lys})_n$-ε-N-DNP-L-lysine (n = 3, 4, 5, 6, or 7) to synthetic polynucleotides, poly (A + U) or poly (I + C).

Interpreting this effect as the result of competition between the cation and the oligolysine for the polynucleotide phosphates, it was possible to group cations into those with a binding preference for poly (A + U) [$(CH_3)_4N^+ > Li^+ > Na^+$; Mg^{2+}, Ca^{2+}, Mn^{2+}]; those with little specificity for either polynucleotide [Lys-H^+, K^+, TrisH^+, NH_4^+]; and those with a binding preference for poly (I + C) [ArgH^+, HisH^+]. Cation specificity for poly (A + U) correlated with cation ability to organize water structure and with volume increase on cation neutralization of polyphosphates, suggesting that specificity in protein-nucleic acid interactions might be directed by the solvent structure surrounding the interacting species. This grouping according to solvent structure promotion also held regarding cation effect on messenger stimulated binding of tRNA to ribosomes, K^+, TrisH^+, and NH_4^+ promoting this binding and $(CH_3)_4N^+$, Li^+, and Na^+ inhibiting it, suggesting a connection between some specific cation effects in biological systems and cation binding to phosphate.

Significance and Proposed Course: The polylysine-polynucleotide system was chosen as a simple model of nucleoprotein interaction. This interaction is known to affect the biological function of the nucleic acid. A study of the nature of the binding forces, stoichiometry, and specificity of this interaction should shed some light on the mechanism by which a basic protein "recognizes" a portion of the nucleic acid structure and affects its properties (repression). Current approaches will be extended to other polyamino acids and nucleic acids. Optical hydrodynamic and biological properties of the complexes will be examined with a view to understanding the geometry of the interaction and the role of the associated structural changes.

Honors and Awards: None

Publications:

Latt, S. A. and Sober, H. A.: Protein-Nucleic Acid Interactions. II. Oligopeptide-Polyribonucleotide Binding Studies. Biochemistry 6: 3293-3306 (1967).

Latt, S. A. and Sober, H. A.: Protein-Nucleic Acid Interactions. III. Cation Effect on Binding Strength and Specificity. Biochemistry 6: 3307-3314 (1967).

Serial No. NCI-386
1. Laboratory of Biochemistry
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Poly-α-amino acids.

Previous Serial Number: Same

Principal Investigators:
A. Herbert A. Sober
B. Elbert A. Peterson

Other Investigators:
A. M. Clyde Otey, Laboratory of Biochemistry
Drs. A. Yaron, A. Berger and E. Katchalski, The Weizmann Institute of Science, Rehovoth Israel and Dr. G. Fasman, Brandeis University, Waltham, Massachusetts
B. Linda Fryklund, Institute of Biochemistry, Uppsala, Sweden

Cooperating Units:
A. Department of Biophysics, The Weizmann Institute of Science, Rehovoth, Israel; Department of Biochemistry, Brandeis University, Waltham, Massachusetts
B. Institute of Biochemistry, Uppsala, Sweden

Man Years:

A.			B.		
	Total:	2		Total:	1
	Professional:	2		Professional:	1/2
	Other:	0		Other:	1/2

Project Description:

Objectives:

A. The preparation and examination of a variety of single and mixed amino acid polymers of known chain length, composition and sequence as model substances for study of the relation between structure of proteins, biological activity, and chromatographic behavior.

B. To extend the applicability of ion exchange electrophoresis to the separation of polyelectrolytes of higher molecular weight.

Methods: Standard as well as newly-designed cellulose ion-exchange adsorbents are used to separate polyelectrolytes by column and thin-layer chromatography, and by ion exchange electrophoresis. The separation process itself is studied by experimental manipulation of adsorbent, salt concentration, electric field, etc. Products resulting from these separation studies serve as model substances in a number of investigations relating to the structure, conformation and interaction of polyelectrolytes.

Major Findings: Lysine oligomers continue to serve as model substances for the study of basic proteins. Oligomers of known chain length, composition and sequence have been prepared and the separation processes have been modified so that individual oligomers of chain lengths as high as 30 can be prepared. They have been used in the following studies:

A. a) The effect of chain length on adsorption to ion exchangers and behavior on Sephadex and polyacrylamide gel-filtration columns.

b) Optical rotatory dispersion studies have been completed. Mathematical computations have been used for analysis of the data and a manuscript is being prepared for publication.

c) Interaction with other polyelectrolytes, primarily polynucleotides and nucleic acids. Details and results are described in NCI-5201.

d) The Merrifield procedure has been used to prepare oligomers of lysine, histidine and ornithine. Chromatographic analysis of the products has indicated that premature termination and cross-linking of chains occur, providing unwanted and spurious products.

B. The use of amphoteric cellulosic adsorbents in thin-layer ion-exchange electrophoresis, as described in last year's report, has been studied further. By adjustment of the pH it was possible to alter the effective number of binding groups on the adsorbent to suit the requirements for light binding of the larger oligomers of polylysine, permitting their migration in a strong electric field. However, conditions have not yet been found that permit simultaneous electrophoretic resolution of polylysine oligomers over a wide range of oligomers, although the range attained is significantly wider than that obtained with CM cellulose.

A column was constructed for high voltage experiments on a larger scale, incorporating means for removal of the migrating bands without interrupting electrophoresis. Separation of oligomers of

DNP-polylysine (yellow in color) could be clearly seen and the bands could be collected, but factors inherent in the method, itself, caused excessive dilution during collection.

Chromatographic tests have demonstrated that the amphoteric adsorbent made with glycine can be used to fractionate polylysine oligomers at pH 5.3, employing a gradient of pyridine acetate for elution, instead of the LiCl or NaCl required for elution from CM cellulose. Since the pyridine acetate can be evaporated readily, the effluent fractions are easily freed of salt.

Significance:

A. Oligolysyl peptides, now available for further studies, are useful model compounds for the investigation of the interactions and functions of basic proteins. Their well-defined structure, size and charge distribution make them valuable as standards for known methods and permit the development of new methods of fractionation, of molecular weight measurement, and of other properties of high molecular weight materials. The polylysine-RNA complexes serve as nucleoprotein models. The use of homogeneous, chemically-defined antigens may permit the study of important problems dealing with the specificity and the nature of antigen-antibody interaction.

B. The concept of controlling the affinity of a cation exchanger by opposing its negatively charged groups with a closely spaced, weakly basic group that can be regulated within the pH range normally used for the chromatography of macromolecules of biological interest is a new and potent one. It fills a gap that has existed because of the lack of monobasic acids with dissociation constants near neutrality. The first practical result is a simpler method of obtaining salt-free oligomers of polylysine, needed for many basic studies on antigenicity, protein structure, and protein-nucleic acid interactions, but applications to the fractionation of other highly charged polycations, such as histones and protamines, are anticipated.

Proposed Course of Study:

A. Repeating polymers of two and three amino acids are being prepared by polymerization of blocked-tripeptides and tetrapeptides in the presence of succinic anhydride. The products, consisting of oligomers of lysine separated by non-basic amino acids, will be analyzed by a variety of procedures and will be used in the studies of protein and nucleic acid interaction.

B. Other amino acids will be incorporated into cellulose in the same manner, or by some modification, and the effect of variation in the spacing of the carboxylate and amino groups will be investigated more rigorously. Similar amphoteric groups will be incorporated into polyacrylamide and agar gels in order to obtain sharper migrating bands during electrophoresis. Relatively low-voltage electric fields will be used to move polycations into electrophoretically stable gradients of increasing pH formed within columns of the amphoteric cellulosic ion exchangers, with the expectation that each component will migrate in a constantly sharpening band to the point in the gradient where tight adsorption prevents further migration. This should provide a high-resolution procedure adaptable to moderately large scale operation.

Honors and Awards: None

Publications: None

Serial No. NCI-320
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the physiological role of catalase and related enzymes and the mechanism of their regulation in normal and neoplastic tissues.

Previous Serial Number: Same

Principal Investigator: Miloslav Rechcigl, Jr.

Other Investigators: Warren H. Evans, Laboratory of Biochemistry, NCI
Walter E. Heston, Chief, Laboratory of Biology, NCI
Harold A. Hoffman, Geneticist, Laboratory of Biology, NCI
Zdenek Hruban, Associate Professor, Department of Pathology, University of Chicago

Cooperating Units: Laboratory of Biology, NCI; Department of Pathology, University of Chicago, Chicago, Illinois

Man Years:

Total:	3-1/2
Professional:	1
Other:	2-1/2

Project Description:

Objectives: To study the alteration in the rates and in the kinetics of synthesis and degradation of catalase and related enzymes, as well as their regulation, under various physiological and pathological conditions, and to investigate the role of these enzymes in metabolism.

Major Findings:

A. The role of enzyme synthesis and degradation - The relative contribution of synthesis and degradation in the control of enzymic levels under various physiological and pathological conditions is still little understood. In contrasting the mammalian with the bacterial systems, it is often pointed

out that the ratio of animal cell life span to enzyme turnover is far greater than in bacteria. As a consequence investigators, rightly or wrongly, have had the tendency of assigning in animal tissues a greater role to the alteration of enzyme degradation than to the enzyme synthesis.

Numerous studies have been carried out in our laboratory on the kinetics of catalase synthesis and destruction in vivo under various experimental conditions, and the results do not agree with the above conclusion; in fact, they seem to imply that the rate of synthesis rather than the rate of degradation, may be the preferential, although by no means the exclusive way of the mammalian organism to control its levels of enzymatic activity. A severe starvation and a lack of dietary protein, both of which evoked a marked decrease in the enzyme levels, while greatly reducing the rate constant for enzyme synthesis, have no apparent effect on the enzyme degradation constant. Similarly, the difference in enzyme activity seen in animals of different ages, as well as those between males vs. females, were all accounted for by the differential rate of synthesis. The heterogeneous activity of various tissues was likewise explained on the basis of differential synthesis of the enzyme. Furthermore, in those neoplastic tissues which have been examined to date, the modification of the rate of enzyme synthesis rather than that of its destruction accounted for the observed differences between the normal and tumor tissues. The species differences, generally, were also due to changes in the rate of enzyme synthesis rather than those in the rate of its degradation. One notable exception with regard to the greater relative importance of synthesis vs. degradation has been found in our studies of enzyme kinetics using two closely related C57BL substrains of mice. In this instance, an alteration in the enzyme degradation constant has been observed - a trait being under a control of a single autosomal gene. For further discussion see below.

B. Studies on biochemical genetics - The finding that different levels of catalase activity in certain of the C57BL substrains are under genetic control provide an excellent model for pursuing fundamental research in biochemical genetics in the mammalian organism. An analysis of the F_1, F_2, and backcross generations between two closely related substrains, the high-liver-catalase substrain C57BL/He and the low-liver-catalase substrain C57BL/6 showed that the difference was due to a single autosomal gene pair with low dominant to high. The recently completed studies on the kinetics of hepatic catalase synthesis and destruction in the two substrains of mice set forth evidence for the gene regulation of enzyme activity by the alteration of the rate constant of enzyme degradation. Linkage studies are now in process to locate and map the liver catalase "regulator" gene. In view of the finding that the dominant low-catalase-gene reduced enzyme activity to only one-half the normal rather than eliminating it completely, and the fact that the

activity of the gene is probably limited to the liver, it appears that the action of the gene may be indirect. Simultaneously, a study is now being made to characterize different inbred strains of mice as to their erythrocyte and kidney catalase levels, and to investigate the mode of enzyme inheritance and possible interaction with genetic and environmental factors. The evidence obtained so far suggests that the enzyme activity in the latter tissues is controlled by different mechanisms than that operating in the case of the liver. Further work is being planned on the enzyme heterogeneity using electrophoretic elution analysis, with particular reference of ascertaining whether or not the observed turnover of the enzyme is limited to a certain molecular species of the enzyme or whether it is a property of all of its isozymes.

C. Studies on the structure and function of microbodies - The almost exclusive presence of catalase, in addition to several hydrogen peroxide-producing oxidases, in the microbodies - the easily identifiable organelles by electron microscopy - has provided an excellent opportunity to study the structure-function relationships of this organelle. This organelle, by some referred to as peroxisome, may play an important role in the metabolism of hydrogen peroxide. In cooperation with Dr. Hruban of the University of Chicago, a number of different tissues of representative species of the animal kingdom are being examined as to their biochemical (enzymatic) makeup and their morphological structure. In general, an excellent correlation seems to exist between the number and structure of microbodies, on one hand, and the activity of urate oxidase, D-amino acid oxidase, and catalase, on the other. In addition to the enzymic activity, the structure of uricase crystals is often discernible by electron microscopy. Interesting observations have been made on the opossum prostate. Histological and structural studies of the prostate of the Philander opossum, Marmosa sp., and Caluromys derbianus revealed the presence of intracellular glycogen and of intraluminal cytoplasmic globules in the cells of the first and third segment. Dense bodies in the first segment of the Philander opossum contains crystalloids. Two types of granules are found in the second segment of all species. They are secreted by two types of cells, except in the wooley opossum. Inclusions with rows of granules are seen in some granules in the second segment of Philander opossum. Biochemical studies show that the first segment of the four-eyed opossum contains large amount of catalase. The third segment in all species has catalase activity several times higher (as much as 15 times) than is observed in the livers of these animals. The cytoplasm of the epithelial cells of the South American opossum does not contain particles which could be interpreted as microbodies. The prosthatic catalase may be localized in the region of microvilli, which in the North American opossum show high peroxidase activity.

Significance to Cancer Research: Since the reduction of catalase activity of the liver and kidney is one of the most prominent features of the tumor-bearing host as well as of porphyria and several other pathological conditions, knowledge of the possible function of this enzyme in the mammalian organism, the mechanism of its regulation, and the kinetics of its turnover are of particular importance to those in the cancer field. Recent reports in the literature on the stimulation of oxidative phosphorylation by this enzyme, coupled with the findings of greatly elevated levels of catalase in certain types of leukemic leukocytes as well as our own evidence implicating catalase in the regulatory capacity over myeloperoxidase activity in phagocytizing leukocytes, suggest that catalase may, indeed, play an important role in the metabolism.

The finding that different levels of catalase activity in certain of the C57BL substrains are under genetic control, provide an excellent model for pursuing fundamental research in biochemical genetics in the mammalian organism. In reference to cancer, the need for genetic markers which can be quickly and quantitatively assayed are extremely important if the genetic control of tumors is to be more quickly elucidated.

Proposed Course of Research: It is planned to extend the studies on the possible role of catalase and related enzymes in the metabolism of leukocytes and morphologically related cells by using specific inhibitors of the enzymes in combination with radioactive tracers. It is also planned to do comparable studies on leukemic cells which have been shown to possess unusually high levels of some of these enzymes. In addition, it is planned to characterize the "leukemic" enzymes and to study their kinetic and cellular distribution. Effort will also be made to relate the functional properties of the enzymes to structural characteristics of the cells and cellular organelles in which they are contained.

Linkage studies are under way in mice to locate and map the liver catalase "regulator" gene. With biochemical genetics in the mouse developing so rapidly this gene should provide an excellent biochemical marker. Studies are also in process, using specific inhibitors of protein and nucleic acid synthesis, to investigate the possibility of the existence of a hypothetical "repressor" and to pinpoint its site of action (i.e., transcriptional or translational level).

A study is now being conducted to characterize different inbred strains of mice as to their erythrocyte catalase levels, and to investigate the mode of enzyme inheritance and possible interaction with genetic and environmental factors.

Further work is being planned on catalase heterogeneity, using electrophoretic elution analysis, in various inbred mice as well as "minimal deviation" hepatomas which are known to vary greatly in their catalase level. It would be of special interest to determine whether different isozymes are "turning over" at identical rates.

Honors and Awards: None

Publications:

1. Rechcigl, M., Jr. and Heston, W. E.: Genetic regulation of enzyme activity in mammalian system by the alteration of the rates of enzyme degradation. Biochem. Biophys. Res. Commun 27: 119-124, 1967.

2. Evans, W. H., and Rechcigl, M., Jr.: Factors influencing myeloperoxidase and catalase activities in polymorphonuclear leukocytes. Biochim. Biophys. Acta 148: 243-250, 1967.

3. Rechcigl, M., Jr.: The rates of synthesis and degradation in the regulation of enzyme levels in animal tissues. Dietologia a dietoterapia 11: 323-358, 1967.

4. Rechcigl, M., Jr. and Price, V. E.: Studies on the turnover of catalase in vivo. In Homburger, F. (ed.): Progress in Experimental Tumor Research. Basel/New York, S. Karger, 1967. Vol. 10, pp 112-132.

5. Rechcigl, M., Jr.: In vivo turnover and its role in the metabolic regulation of enzyme levels. Enzymologia 34: 23-39, 1968.

Serial No. NCI-366
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Structural and biochemical investigations of intracellular components.

Previous Serial Number: Same

Principal Investigator: Edward L. Kuff

Other Investigators: Emma Shelton, Kira Lueders, John T. Harrington

Cooperating Units: None

Man Years:

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives: Investigation of the cytochemical organization of normal and malignant cells, with particular reference to the reduplication of intracellular components. Present studies are centered on the physical and functional properties of cytoplasmic ribonucleoproteins.

Methods: Tissue fractionation by differential and density gradient centrifugation, isolation and gradient fractionation of nucleic acids and nucleoproteins, electron microscopy of isolated nucleoproteins, in vivo and in vitro incorporation of isotopically-labelled precursors into protein and nucleic acid, chromatographic and immunochemical assay of specific proteins.

Results: A. Previous annual reports of this project describe our findings with respect to the structure of plasma-cell tumor polyribosomes and their functional capacities in protein synthesis. In the period covered by the present report, we have been attempting to define more clearly the relationship of the ribosomal units to the messenger RNA.

1. Polyribosomes isolated from tumors following a brief (20 min.) in vivo incorporation of H^3-uridine contain labelled RNA which is heterogeneous on sedimentation analysis and corresponds to neither the ribosomal nor transfer RNA's. This rapidly labelled RNA was assumed as a working hypothesis to represent wholely or in part the messenger RNA components of the polyribosomes.

2. Polyribosomes have been treated with EDTA in amounts sufficient to dissociate the component ribosomes, and the resultant mixtures analyzed by centrifugation on sucrose density gradients. The rapidly-labelled RNA is contained in structures with a wide range of sedimentation velocities, for the most part moving more rapidly than the ribosomal subunits.

3. These EDTA-released structures, containing RNA of very high specific radioactivity relative to that of the original polyribosome fraction, have been examined by electron microscopy (for details, see Annual Report Serial No. NCI-379, by Dr. Shelton). They consist of thread-like elements 20-30 Å in thickness and up to 1200 Å in length, along which particulate components approximately 200 Å in diameter are disposed at apparently irregular intervals.

4. The precise composition of these structures is not known. It is our tentative conclusion based on labelling data, electron microscopy, and buoyant density determinations, that they represent complexes of messenger RNA with protein and/or lipid material, in association with a few ribosomal subunits which have failed to dissociate from the complex upon EDTA treatment.

5. Morphologically similar elements can be released from purified tumor cell nuclei by mild extraction procedures, without recourse to chelating agents. The nuclear-derived particles resemble those released from the cytoplasmic polyribosomes by EDTA in certain respects (content of rapidly labelled polydisperse RNA, similar buoyant density in CsCl gradients) and this relationship is under current investigation.

Significance to Cancer Research: Recent results from several laboratories suggest that messenger RNA emerges from the nucleus complexed with protein. The formation of functional polyribosomes thus involves the successive attachment of ribosomes to a messenger RNA-protein complex (which apparently persists within the polyribosome and can be released by EDTA treatment). The specific association of messenger RNA with protein during nucleo-cytoplasmic transport and translation could constitute a major regulatory mechanism for intracellular synthesis.

Future Plans: Studies will be continued aimed at elucidating the mechanism of transport of mRNA from nucleus to cytoplasm and the incorporation of mRNA into functional polyribosomes.

Honors and Awards: None

Publications:

1. Kuff, E. L., and Roberts, N. E. : In vivo labelling patterns of free polyribosomes: Relationship to tape theory of messenger RNA function. J. Mol. Biol. 26: 211-225, 1967.

2. Lueders, K. K., and Kuff, E. L. : Spontaneous and detergent activation of a glucuronyl-transferase in vitro. Arch. Biochem. Biophys. 120: 198-203, 1967.

3. Cummings, N. A., Kuff, E. L., and Sober, H. A. : Examination of magnesium binding to serum proteins by ultracentrifugal analysis. Anal. Biochem. 22: 108-116, 1968.

4. Kuff, E. L., Wivel, N. A., and Lueders, K. K. : The extraction of intracisternal A-particles from a mouse plasma-cell tumor. Cancer Res., in press, 1968.

Serial No. NCI-368
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Perfusion of isolated tumors.

Previous Serial No.: Same

Principal Investigator: Pietro M. Gullino

Other Investigators: F. H. Grantham and I. Losonczy

Cooperating Units: None

Man Years:

Total:	3
Professional:	1/2
Other:	2-1/2

Project Description:

Objectives: This work is part of a study on the conditions which may produce a modification in vivo of biological properties of a tumor. In the past year we learned to perfuse a tumor as an isolated organ detached from the host. The reasons for developing such a system are two-fold: a) The necessity of separating the response of the host from that of the tumor in an assessment of the effects in vivo of substances acting on the neoplastic tissue, and b) the need of maintaining the anatomical integrity of the tumor in order to evaluate the physiological meaning of any "response" elicited by substances active on neoplastic cells maintained in artificial media.

Methods: The starting point of the work was the tissue-isolated tumor, a preparation developed a few years ago (see previous reports). As a first step a surgical procedure was developed for the isolation of the vascular peduncle of the tumor and the efficient cannulation of the vessels. As a second step, a circuit was assembled which enabled us to perfuse the tumor in the simplest possible way. The circuit is formed by 2 pumps, a "minilung" with a system for pulsatile flow, a microchamber for continuous pH determinations, a dialyzing system and 2 containers, one for the tumor and the other for the perfusate. Three perfusates were used, heparinized total blood, blood diluted with Eagle's medium 2:1 and an erythrocyte-free perfusate formed by Eagle's medium + rat serum (20%) + 7 gm % of bovine albumin.

Findings: 1) The flow-pressure curve of the tumors perfused with total or diluted blood has a shape which is peculiar to the neoplastic tissue and it is formed by 3 components. The first is characterized by a sharp increase in

resistance to flow, regardless of the flow rate. Despite a large amount of effort, we are still unable to understand the reasons for the initial hypertensive phase. The second part of the curve is characterized by a disassociation between flow and pressure. Within certain limits, it is possible to double the flow through the tumor with very little increase in pressure. The third part of the curve concerns the beginning of the end of perfusion and manifests itself with hypertensive crisis followed by irregular changes in the flow until no perfusate can flow through the tumor despite a very high pressure. Perfusates without erythrocytes do not present this last hypertensive phase and perfusion can last for days.

2) The ability of mammary carcinomas to extract O_2 from the afferent blood was as good in perfused tumors as *in vivo* provided that total blood was used. When blood was diluted about 30% with Eagle's medium or saline the ability of the tumor to utilize O_2 from the perfusate was reduced by a factor of 2.3 despite an O_2 availability reduction of only 22%. Dilution of blood *per se* impairs the oxygen removal capacity of the tumor. When erythrocyte-free perfusate was used, the amount of O_2 utilized dropped to values 25-fold lower than the level measured for total blood. The conditions underlying this finding were defined as follows: The erythrocyte-free perfusate was carried to the tumor with a pO_2 = 145-150 mmHg (equal to the pO_2 of arterial blood 95% oxygenated) and left the tumor with a pO_2 = 90-100 mmHg (equal to the pO_2 of venous blood 65% saturated). For the tumor, the erythrocytes represent a supply of O_2 which was released during the passage through the neoplastic vessels. Lacking this reservoir, the tumor was unable to lower the O_2 content of the fluid phase of the perfusate below a pO_2 - 90 mmHg. Therefore the O_2 consumption of Walker carcinomas, for instance, fell from 1.96 to 0.8 mmole/hr/100 gm and the limiting factor to an increase of O_2 supply was the amount of fluid which could be passed through the tumor.

3) The ability of mammary carcinomas to remove glucose from the afferent blood was equal or larger than *in vivo*. The total consumption, however, was smaller than that *in vivo* because the maximal amount of blood which could be perfused *in vitro* was smaller than the flow measured *in vivo* when the glucose uptake was calculated. A surprising dissociation between O_2 and glucose consumption was observed. If a Walker carcinoma, for instance, was perfused first with the erythrocyte-free perfusate and then erythrocytes were added (27% hematocrit; 9.0 gm% Hb) a 10-fold increase in O_2 consumption was observed but no appreciable change in glucose utilization.

4) Walker carcinomas perfused with total blood transformed about 50% of the utilized glucose into lactate. This was higher than the value *in vivo* (35%). If blood was diluted or erythrocyte-free perfusate was used, about 80% of the utilized glucose was transformed into lactate. There was practically no difference in lactate production between tumors perfused with perfusates with or without erythrocytes despite a 10-fold reduction in O_2 utilization observed with erythrocyte-free perfusates.

5) Long term perfusions (more than 24 hours) with erythrocyte-free perfusate were unsuccessful. Walker carcinomas perfused for 50-60 hours with perfusates

having a 7% hematocrit (about 2 gm Hb%) survived and were transplantable. A satisfactory evaluation of growth is not yet available.

Significance: Work on the physiopathology of tumors has been traditionally carried on using 4 experimental systems: tumor+host, fragments of neoplastic tissue surviving in various media, neoplastic cells in culture and subcellular particles. If one considers the large amount of information gained from the study of intact organs surviving separated from the organism, it seemed necessary to develop a fifth experimental system i.e. a perfused isolated organ in which the tumor could be separated from the host but kept intact with the tissue components in physiological relation.

Proposed Course: Determination of uptake and release of substances which may change the biological properties of mammary tumors.

Honors and Awards: None

Publications:

1. Gullino, P. M., Grantham, F. H., and Courtney, A. H.: Glucose consumption by transplanted tumors in vivo. Cancer Res. 27: 1030-1040, June 1967.

2. Gullino, P. M., Grantham, F. H., and Courtney, A. H.: Utilization of oxygen by transplanted tumors in vivo. Cancer Res. 27: 1020-1029, June 1967.

3. Gullino, P. M., Grantham, F. H., Courtney, A. H., and Losonczy, I.: Relationship between oxygen and glucose consumption by transplanted tumors in vivo. Cancer Res. 27: 1041-1051, June 1967.

Serial No. NCI-379
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on cell interaction and differentiation.

Previous Serial Number: Same

Principal Investigator: Emma Shelton

Other Investigators: Edward L. Kuff

Cooperating Units : None

Man Years:

Total:	6
Professional:	3
Other:	3

Project Description:

Objectives: To study the fine structure of ribosomes and related structures.

Methods: Electron microscopy and related techniques.

Major Findings:

1. Discovery of a "new" cell structure.

In the course of a project in collaboration with E. L. Kuff designed to study the structure of naturally occurring ribosomal subunits, a hitherto undescribed cellular entity was discovered. When cytoplasmic extracts were centrifuged in a 15-30% sucrose gradient, the particles were found in a fraction representing a sedimentation coefficient of approximately 32 S. They have a characteristic morphology that permits their unequivocal detection in the electron microscope. Each particle consists of a "head" and a "body". The head is a single structure which has a characteristic trefoil shape when attached to the body while free heads appear to have four lobes. The body is made up of four identical doughnut-shaped cylinders, which when viewed on edge look like a stack of coins. The stack of four units may have a head attached to one or both ends; however, it appears that at least half of the bodies have no attached heads. Dialysis against buffer causes the stack to fall apart revealing the hole in the middle of the cylinders.

Approximate dimensions of the bodies are 100 x 150 Å and of the heads 135 x 200 Å; the length of the complete particle is approximately 185 Å.

These particles have been identified in two plasma cell tumors of the mouse, in mouse liver, in rabbit reticulocytes, in human erythrocytes and in pea seedlings. So far, they have not been identified in bacteria despite an intensive search.

The nature of the particles is unknown. They seem to resist digestion with deoxyribonuclease, ribonuclease, phospholipase A, C and D but are sensitive to pronase. It is tentatively proposed that they represent an enzyme consisting of four subunits (the body) and its substrate (the head).

Purification of the particles, which are grossly contaminated with soluble proteins and ribosomal subunits, is hampered by lack of a rapid assay. So far the only assay is identification by electron microscopy.

2. Fine Structure of ribosomal subunits.

Examination of naturally occurring ribosomal subunits and of subunits released from polyribosomes by EDTA treatment has yielded provocative electron micrographs. When exposed to uranyl ions, these ribosome subunits exhibit a common basic infrastructure of an amorphous background interspersed with densely stained 5-8 Å spots or clusters. These densely stained areas probably represent local concentrations of RNA. In addition to this basic infrastructure, a circular area of approximately 180 Å in diameter has been observed in the naturally occurring 45 S subunits.

Uranyl acetate positive staining of ribosomes does not permit an accurate assessment of particle shape; all of the subunits appear to be roughly spherical or elliptical with ill-defined edges and, with the exception of the 60 S subunit, the particles appear to be relatively compact. It is of considerable interest, then, that 60 S subunits released from polyribosomes consistently exhibit 20 Å filaments "attached" to their surfaces. Usually only one such filament can be observed per subunit, but as many as three have been seen. The length of the filaments varies from 75-400 Å. They may represent an "uncoiling" of the ribonucleoprotein of the subunit, but it is odd that filaments very rarely occur on the 40 S subunit released at the same time or on the naturally occurring 45 S subunits.

3. Fine structure of rapidly labeled material released from polyribosomes.

When polyribosomes from cells labeled for 20 minutes with H^3 uridine are treated with EDTA and sedimented in a 20-60% sucrose gradient, a large proportion of the radioactivity sediments in a region heavier than the released ribosomal subunits (see Annual Report, E. L. Kuff No. 366). Examination of the material reveals 20-50 Å strands some of which are several thousand angstrom units in length. These strands are not single

filaments since they can be seen to divide into 2 or more strands at intervals along their length. Neither are they straight since they may branch and zig-zag. At intervals along the length of these strands, distinct circular areas can be seen. Some of these are open circles, some are more dense; their diameter is roughly 175-200 Å. Similar structures are observed when isolated polyribosomes are examined within 10 minutes after treatment with sufficient EDTA to release ribosome subunits from the messenger RNA to which they are attached. Whether the circular areas dispersed along the length of the complex filaments represent ribosomes attachment sites or parts of ribosomes remaining attached to the strands is at present a matter for conjecture.

Proposed Course of Study: To elucidate the relationship between fine structure of cells and their functional transformation.

Importance to Medical Research: The mechanism by which cells alter their metabolism in response to new environments are poorly understood. Increased knowledge of such mechanisms may provide insight into those metabolic changes that lead to the malignant alteration.

Honors and Awards:

1. Invited speaker: New York Society for Electron Microscopy and New York University School of Medicine Symposium "Views and Models of Biomacromolecules", New York City.

2. Lecturer: Department of Biophysics, Johns Hopkins University, Baltimore, Maryland.

3. Lecturer: Senior Medical Class Colloquium, Downstate Medical Center, S. U. N. Y. Brooklyn, New York.

4. President, Washington Society for Electron Microscopy.

5. Member, Editorial Advisory Board, Cancer Research.

Publications:

1. Greenblatt, C. L., and Shelton, E.: Mouse serum as an environment for the growth of Trypanosoma lewisi. Exp. Parasit. 22: 182-200, 1968.

Serial No. NCI-5206
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of Protein Synthesis.
I. initiation of protein synthesis
II. peptide bond formation
III. translocation reaction; motion of mRNA relative to ribosome
IV. characteristics and function of ribosomal binding sites
V. deformylation reaction

Previous Serial Number: Same

Principal Investigator: Philip Leder

Other Investigators: Richard W. Erbe and David M. Livingston

Cooperating Units: None

Man Years:

Total:	4
Professional:	3
Other:	1

Project Description:

Objectives: To define the molecular mechanism which initiates protein synthesis, initial and subsequent peptide bond formation, which provides for the orderly translocation of messenger RNA across the ribosome during protein synthesis and for the removal of the initial blocking group from the synthesized peptide.

Methods Employed: Various chemical and enzymatic procedures have been employed to synthesize and characterize small oligonucleotide messenger RNA molecules and to prepare appropriate aminoacyl-tRNA's. Subcellular elements, ribosomes, have been prepared from E. coli and systems established in which codon recognition and peptide bond formation may be studied. In addition, factors required for the correct initial and subsequent translation of mRNA have been isolated, principally by ion exchange chromatography.

Results: Rapid assay methods have been developed which permit individual detection of translation of the first and second codons of dicodon messengers, and translocation of mRNA relative to the ribosome. The use of the small messenger system and these specific assays has permitted us to define the function of isolated factors required for protein synthesis. Of these, at least two together with GTP, are required for the correct initiation of protein synthesis. Translation of the second codon requires the addition of a third factor, involved in a binding interaction, and is accompanied by formation of the appropriate dipeptide. Further translation of a tri-codon requires the addition of yet another factor, indicating its involvement in the translocation process.

Further studies have shown that the formyl blocking group of N-formyl-methionine-tRNA, which is required for correct initiation of protein synthesis in bacterial systems, is removed by an enzyme partially purified from E. coli. Specificity of this enzyme requires that the formyl-methionine be incorporated into peptidyl-tRNA prior to deformylation. This result is consistent with the role of the formyl group in initiation of protein synthesis and its redundancy after formation of the first peptide bond.

Significance and Proposed Course of Research: The results permit a functional definition of factors involved in correct translation of mRNA and provide a well-defined system in which this translation may be studied. We intend to explore the mechanism by which proteins are correctly assembled on ribosomes, to define the site of specific actions on the ribosomal subunits, and to study the effect of limited and extensive translation of mRNA on transcription, a point at which general regulation of protein synthesis may occur.

Honors and Awards: None

Publications:

1. Leder, P., and Nau, M. M.: Initiation of protein synthesis. III. Factor-GTP-codon-dependent binding of F-met-tRNA to ribosomes. Proc. Nat. Acad. Sci. 58: 774, 1967.
2. Leder, P.: Enzymatic synthesis of trinucleoside diphosphates of known sequence. In: Methods in Enzymology, vol. XII, Grossman, L. and Moldave, K. (eds.) 1968, Academic, New York, In press.
3. Leder, P.: An assay for the binding of C^{14}-aminoacyl-tRNA to ribosomes. In: Methods in Enzymology, vol. XII, Grossman, L. and Moldave, K. (eds.) 1968, Academic, New York, in press.

Serial No. NCI-5208
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Hyaluronic acid content and hyaluronidase activity of tumor interstitial fluid.

Previous Serial No.: None

Principal Investigator: B. Fiszer-Szafarz (guest worker)

Other Investigator: Pietro M. Gullino

Cooperating Units: None

Man Years:

Total:	2
Professional:	1-1/4
Other:	3/4

Project Description:

Objectives: This project is part of a study on conditions which may influence the in vivo transfer of substances to neoplastic cells. It is generally accepted that long molecules of hyaluronic acid (HA) are distributed in the interstitial spaces of tissues to form a web. Some experiments indicate that the density of this three dimensional net plays a major role in regulating the transfer of substances from the vascular to the cell compartment (T. Day; J. Physiol. 117: 1, 1952). This work aimed at finding:

a) The HA content of the interstitial fluid of a tumor (TIF) and compare it with the concentration in the subcutaneous interstitial fluid (SIF) and serum of the host.

b) The possible difference between TIF and SIF in the degree of polymerization of HA.

c) The level of hyaluronidase activity (Hase) of TIF, SIF and serum since it can influence HA polymerization.

Methods: HA content was measured with a turbidimetric method before and after treatment with H'ase (Bull. Soc. Chem. Biol. 46: 1211, 1964). The degree of polymerization of HA was analyzed with a method based on differential elution of glycosoaminoglycans from a cellulose column (B.B.A. 83: 1, 1964). The H'ase activity was measured by the increase in the amount of acetylglucosamine in an assay system containing HA (Bull. Soc. Chem. Biol.

46: 1211, 1964). The interstitial fluid was sampled with a method developed in this laboratory (previous reports).

Results: 1) The average content of HA in the interstitial fluid of the sacral subcutaneous region was 38.9 µl/ml but the value for TIF of Walker carcinoma transplanted in the same region was 21.6 µg/ml. The presence of a 10 gm tumor was sufficient to effect a decrease of HA concentration in the SIF of regions distant from the neoplastic mass. In the scapular region, for instance, values changed from 67.8 µg/ml in normal to 34.0 µg/ml in tumor bearing animals.

2) The SIF of the scapular subcutaneous region contains chondroitin sulfuric acid and 2 fractions of HA, one with a higher degree of polymerization than the other. TIF of Walker carcinomas transplanted in the scapular region does not contain any chondroitin sulfuric acid and has only the low molecular fraction of HA.

3) The H'ase activity of SIF is about 1/4 that of the normal serum; H'ase activity of TIF is about 1/2 that of the host serum, and about 1/3 higher than that of SIF; H'ase activity of the serum of tumor bearing hosts is about 1/2 the activity of normal serum.

Significance: We have collected some basic data in an area of cancer research where very little is known. The results are useful for the next step of this work: whether it is possible to change in vivo the HA content of TIF and induce as a consequence, a change in the transfer of macromolecules from the vascular into the interstitial compartment.

Proposed Course: As stated in the previous paragraph.

Honors and Awards: None

Publications: None

Serial No. NCI-5209
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The uptake of DNA from lactating mammary glands by mammary carcinomas.

Previous Serial No.: None

Principal Investigator: C. Watters (visiting scientist)

Other Investigator: P. M. Gullino

Cooperating Units: None

Man Years:

Total:	1-1/2
Professional:	1-1/4
Other:	1/4

Project Description:

Objectives: 1) To determine the rate of DNA uptake by mammary tumors *in vivo* and *in vitro*.

2) To investigate if any biological effect results from the treatment.

Methods: 1) Mammary gland DNA preparation by the phenol extraction procedure from thymidine-methyl-H^3 injected rats in the first week of lactation.

2) Separation and analysis of various fractions of the DNA preparation by elution chromatography on cellulose column (Progress Nucleic Acid Res. 4: 231, 1965).

3) Perfusion of tumors *in vivo* and *in vitro* according to techniques developed in our laboratory (see previous reports).

4) Analysis of DNA uptake by fractionation of the perfused tumor according to Schmidt-Thannhauser (modified) (Analyst 86: 768, 1961).

Major Findings: 1) DNA added to total or diluted blood can be perfused through a mammary tumor for 5-6 hours without any major change in the molecular weight profile.

2) The amount of DNA taken up by the erythrocytes during 5-6 hours of perfusion was negligible.

3) The uptake of DNA by Walker carcinomas is in the order of 0.3% of the total tumor DNA after 6 hours of perfusion.

Significance: These experiments represent the initial phase of an attempt to analyze the biological effect of the DNA extracted from a lactating mammary gland upon mammary carcinomas.

Proposed Course: 1) Find out ways of increasing uptake; 2) detect the kind of biological activities of the tumor which may be changed by DNA.

Honors and Awards: None

Publications: None

Serial No. NCI-5210
1. Laboratory of Biochemistry
2. Biosynthesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanism of action of steroid hormones in inducing enzymes in hepatomas in tissue culture.
Previous Serial Number: None
Principal Investigator: E. Brad Thompson

Other Investigators: None

Cooperating Units: Dr. Aaron Shatkin, Laboratory of Biology of Viruses, NIAID; Dr. Harold Moses, Laboratory of Experimental Pathology, NIAMD; Drs. Gordon M. Tomkins, D. K. Granner and T. D. Gelehrter, Laboratory of Molecular Biology, NIAMD; Drs. Isidore Edelman, Rita Bogorach and David Inman, Cardiovascular Research Unit, University of California Medical School at San Francisco, and Dr. Alex Kohn, Israel Institute for Biological Research.

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

Objectives: To elucidate at a molecular level the mechanism of action by which steroid hormones induce enzymes in hepatomas, and to relate this to normal and malignant cells in general.

Methods Employed and Major Findings: Morris hepatoma no. 7288C has been adapted to tissue culture and this tissue culture line has been used for all this work. We have previously shown that steroid hormones with glucocorticoid action induce an increase in activity of the enzyme tyrosine aminotransferase in this cell line. Preliminary work suggested that this increased activity was due to new protein synthesis and that continued induction required the continued presence of the inducing steroid. The

regulatory mechanisms involved have been pursued using a variety of methods, including radioautography, electron and light microscopy, biochemical methods, viral fusion of cells, and immunological techniques.

Microanatomic studies with Drs. Edelman, Bogorach, Inman and Moses have been begun using radioautography to study the localization of steroid within the cell, seeking its anatomic site of action. Preliminary results show this to be perinucleolar. Also, studies have been started seeking to determine the effect of steroid on the intracellular sites of DNA and RNA synthesis.

Using a radioimmunological technique it has been shown that tyrosine aminotransferase induction is due to an increase in the rate of synthesis of the enzyme and that upon removal of inducer the synthetic rate returns toward basal levels. It has also been shown that the rate of synthesis increase precedes the accumulation of enzyme activity upon induction.

Blockers of macromolecular synthesis have been employed to study this system. Experiments with Actinomycin D and other agents have suggested that the messenger RNA for tyrosine transaminase is relatively long-lived and that it is under control at the level of translation as well as at the level of transcription from DNA.

Experiments attempting to fuse using certain killed viruses, the inducible HTC cells to other, non-inducible cells have been begun.

From these experiments, a model to explain the control mechanisms involved in enzyme induction in mammalian cells has been proposed. The novel aspect of this model is that it states that there is control at two levels, at the level of gene transcription as in bacteria and also at the level of messenger RNA translation.

Separately from the above, a review article has been prepared concerning the biochemistry of the lipids and proteins of brain white matter.

Significance to NCI Research and Proposed Course of Study:

Understanding the regulatory mechanisms involved in mammalian cell protein synthesis, and the mechanism by which steroid hormones influence these controls should be of importance to general biological theory as well as to understanding clinical situations in which steroid hormones are altered in type or quantity in the patient. Since this work is done in hepatomas, comparison with normal cell control systems may provide better understanding of the cancer process as well as the normal cell. It is proposed that the experiments begun be pursued.

Honors and Awards: None

Publications:

Tomkins, G. M., Gelehter, T. D., Granner, D. K., Peterkofsky, B., and Thompson, E. B. : Regulation of gene expression in mammalian cells. 22nd Ann. Symp. on Fundamental Cancer Research, M. D. Anderson Hospital and Tumor Institute, in press, 1968.

Thompson, E. B. : In Handbook of Clinical Neurology. Biochemistry of the proteins and lipids of white matter. Vinken, P. J. and Breuyn, G. W. (Eds.) North-Holland publishing Co., Amsterdam, in press 1968.

Serial No. NCI-330
1. Laboratory of Biochemistry
2. Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Combined actions of vitamins, peroxides, anti-catalases and moderate hyperthermy on cancer metabolism and growth.

Previous Serial Number: NCI-330 (plus 1965-1966) NCI-331, 332 and 334.

Principal Investigators: Dean Burk and Mark Woods

Other Investigators: Tulane Howard, and Leonard Benade

Cooperating Units: O. Warburg, Max Planck Institute, West Berlin, Germany
M. von Ardenne, Ardenne Institute, Dresden, Germany
H. Schirmer, Johns Hopkins Hospital, Baltimore, Maryland
R. Madden, Fifth Avenue Hospital, New York, New York
J. Dittman, Homburg Hospital, Saar, Germany
K. Sanford, Tissue Culture Section, Laboratory of Biology, NCI
W. Heston, Chief, Laboratory of Biology, NCI.

Man Years:

Total:	4-1/2
Professional:	3
Other:	1-1/2

Project Description:

Objectives: To determine the action and mode of action of chemical and physical anti-cancer agents on the metabolism, growth and death of cancer cells in vitro or in vivo. Particular emphasis is placed on relatively non-toxic vitamins, (e. g., riboflavin and vitamin C), moderate hyperthermy, and potentiators thereof.

Methods: Tumor materials are exposed either in vitro or in vivo to chemical or physical (e. g., hyperthermic) agents, and effects on metabolism, growth or death subsequently determined. Methods of metabolic analysis include

the use of several series of tumors (C_3H and $C3H\text{-}A^{vy}$ spontaneous mammary tumors, S91 Mouse melanomas, Ehrlich ascites tumor, Morris rat hepatomas, Sanford tissue culture cell lines) exhibiting a spectrum of hormonal sensitivities, as well as clinical materials as tumor test systems. In vivo effects on tumor growth and viability are measured with appropriate biological techniques, or after removal of tumor tissues, biochemical techniques including manometry and spectrophotometry.

Major Findings:

Hydrogen peroxide generators: Oxidation of certain vitamins (e.g., B_2, K_5) in cell-free media yielded hydrogen peroxide. If the system contained catalase, H_2O_2 accumulation was prevented. When very small amounts of copper sulphate (less than 1 part per billion of Cu^{++}) were added to the medium, oxidation of ascorbate (Vitamin C) was greatly increased. Addition of catalase inhibited this oxidation of vitamin both in the presence and absence of added copper. It could be shown spectrophotometrically (with formation of a new band at about 440 mμ) that catalase combines with copper ions. However, the hydrogen peroxide-splitting function of the enzyme was not blocked by copper, at least at concentrations that affected ascorbate auto-oxidation. Presumably catalase inhibits the "auto-oxidation" of ascorbate to which no copper is added by complexing with amounts of copper present as an unintended contaminant.

Ehrlich ascites cancer cells were exposed aerobically to ascorbate plus and minus amino-triazole. High levels of ascorbate alone produced some cell death (determined by measurement of respiration and/or glucolysis, and trypan blue staining). Amino-triazole enhanced the lethal action of ascorbate, presumably by inhibition of the already weak catalase activity of Ehrlich ascites tumor cells. In vivo tests of the action of these agents are under way. In vivo effects comparable in magnitude to those seen in vitro have not been obtained as yet, but factors such as dosage, glucose concentration, intratumoral oxygen pressure, etc., have yet to be optimalized. An important aspect of potential advantage in the use of agents such as ascorbate, amino-triazole, riboflavin, etc., is their extremely low host toxicity (LD_{50} = 10-20 g/kg!), which permits very high dosages to be given in vivo. Presumably, the lethal effects on cancer cells of such vitamins (C, B_2, etc.) derive in part from increased respiratory abnormalities and/or low catalase content of the tumor cells. The effects of adjunctive agents (e.g., copper) on host toxicity of otherwise non-toxic vitamins (e.g., C, B_2) are being determined.

Moderate hyperthermy: Von Ardenne and Kirsch of Dresden introduced early in 1965 the clinical treatment of cancer patients at uniformyl maintained, extreme body temperatures of about 42-44°C for

periods of time ranging up to 35-45 minutes. Recently these workers have shifted emphasis to moderately hyperthermic temperatures (40-42°) with agents (studied first in experimental animals) which selectively augment the therapeutic action of this moderate hyperthermy. We have followed two main lines of attack on this problem, (a) development of the use of agents to materially lower the heat tolerance of the tumor without materially decreasing the heat tolerance of the host animal and (b) development of chemical or physical treatments that will increase the heat tolerance of the host animal without increasing the thermal tolerance of the tumor. Ideally, (a) and (b) would be combined. Current work involves tests in mouse tumor systems of various potential anti-cancer vitamins combined with moderate hyperthermy and adjunctive agents (e. g., anti-catalases).

Vitamins and hyperthermy of spontaneous tumors. Female C_3H and C_3H^{vy} mice show a high incidence of mammary cancer (Heston) and male C_3H^{vy} mice a high incidence (90-100%) of liver cancer. In collaboration with Dr. Heston and Mr. Vlahakia, weanling mice of the C_3H^{vy} strain are being exposed to stressful air temperatures of 36°-37° C (rectal temperatures remaining normal or nearly so (37-38°C). The effects of such mild "hyperthermy" on tumor incidence will be determined. In other tests with C_3H^{vy} mice the effects of feeding high levels of riboflavin (vitamin B_2) and dried Brewer's yeast on tumor incidence (mammary tumors in females and hepatomas in males) will be studied, following much early work (Sugiura, Rhodes, et al.,), clearly demonstrating that such dietary supplements can give marked to complete protection against liver cancers in rats induced by "butter yellow" (p-dimethylaminoazobenzene).

Honors and Awards: None

Publications:

1. Burk, D., and Woods, M.: Newer aspects of glucose fermentation in cancer growth and control. Arch. Geschulstforschung 28: 305-319, 1967.
2. Burk, D., and Woods, M.: Glycolysis. In Williams, R. (Ed.), Encyclopedia of Biochemistry. Reinhold, New York, New York, 1967, pp. 210-220.
3. Burk, D., Woods, M., and Hunter, J.: On the significance of glucolysis for cancer growth, with special reference to Morris rat hepatomas. J. Nat. Cancer Inst. 38: 839-863, 1967.

4. Woods, M., Burk, D., and Hunter, J.: Factors affecting anaerobic glycolysis in mouse and rat liver and in Morris rat hepatomas. J. Nat. Cancer Inst., in press 1968.

5. Warburg, O., and Burk, D. (Editors): The prime cause and prevention of cancer. Triltsch, Wuerzburg, November 1967, pp 1-16.

6. Burk, D., Woods, M.: Hyperthermy-chemotherapy of mouse melanoma and Ehrlich ascites cancer cells. Proc. 5th Inter. Cong. Chemother. (Vienna), Vol. II, Pt. 1, pp. 259-262, 1967.

Serial No. NCI-334
1. Laboratory of Biochemistry
2. Cytochemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The occurrence and function of a stress-modifiable anti-insulin hormonal complex regulating glucose utilization in cancer and normal cells and its relation to chemotherapy at normal and hyperthermic temperatures.

Previous Serial Number: Same

Principal Investigator: M. Woods and Dean Burk

Other Investigators: Tulane Howard and Eleanor Hall

Cooperating Units: K. Sanford, Tissue Culture Section, Laboratory of Biology, NCI; H. Morris and B. Wagner, Nutrition and Carcinogenesis Section, Laboratory of Biochemistry, NCI; E. P. Vollmer, Chief, Endocrine Evaluation Branch, NCI; Dr. W. Heston, Chief, Laboratory of Biology, NCI; and Mr. George Vlahakis, Laboratory of Biology, NCI.

Man Years:

Total:	3-3/4
Professional:	3
Other:	3/4

Project Description:

Objectives: To determine the extent to which insulin:anti-insulin hormonal regulation of glucose metabolism functions as a metabolic control mechanism in cancer and normal mammalian cells, and to determine ways in which this mechanism can be manipulated as an adjunct to in vivo therapy including possible involvement of such hormonal metabolic regulation in host defensive cells (RES), and in exposure of cancer cells to hyperthermic temperatures (40° - 45° C).

Methods: The responses of tumor and normal tissues are determined in vitro and in vivo with respect to anti-insulin inhibition of glycolysis (e.g., by steroids, stilbestrol, Podophyllum derivatives (podophyllotoxin, alpha and beta peltatins, fraction CC7E, etc), and to substances which counteract this inhibition (insulin, bacterial endotoxins). The action of these, and other chemical agents on cancer and normal cells at elevated temperatures (40-45°C) are determined with reference to cancer-host differential responses. Various tumors or normal tissues presenting a known spectrum of sensitivities to insulin:anti-insulin regulation, as well as clinical tumors of unknown

sensitivities, are employed in these studies.

Major Findings: Further confirmation and extension of the anti-glucolytic properties of Δ^1-testololactone (TL; NSC-23759) were obtained in spontaneous mammary tumors of C_3H and C_3H^{vy} mice (Heston). Direct in vitro insulin-induced stimulation of glucolysis, and conversely, inhibition of glucolysis by natural anti-insulin steroids (testosterone, estradiol), were also obtained in these mammary tumors. Individual mammary tumors from the same host animal showed highly significant differences in glucolytic rates ($Q_{CO_2}^{N_2}$) response to insulin and anti-insulin hormones. While these mammary tumors differed from each other with respect to metabolic resistance to in vitro hyperthermy (43-45° C), as a group they were much more resistant to hyperthermic destruction of aerobic metabolism (respiration and the Pasteur effect) and of glucolysis (aerobic and anaerobic) than was the case in S91 mouse melanoma or Ehrlich ascites tumor cells. For example, at 45° C the time required to completely destroy the Pasteur effect (point at which oxygen no longer has any depressive effect on glycolysis, i.e., Pasteur effect = zero) was 35 minutes in the S91 malnoma, 80 minutes in the Ehrlich ascites tumor, and over 135 minutes in the C_3H mammary tumor.

An active glucose metabolism in all tissues studied enhanced cell resistance to the destructive effects of hyperthermy. In general, impairment of glucolytic metabolism (e.g., by steroids) promoted tumor cell death by hyperthermy and stimulation of glucolytic metabolism (e.g., by insulin and/or increased glucose level) retarded death by hyperthermy. Agents which enhance cell susceptibility to hyperthermy (e.g., steroids, 2,4-DNP, serotonin, etc.) can be readily identified by measuring their ability to shorten the time required at any given temperature to eliminate the Pasteur effect. Agents which protect the tissue from hyperthermic injury increase this "critical time" required for elimination of the Pasteur effect. Exposure to anoxia, glucose deprivation, and especially both together (= simulated ischemia) greatly increased the susceptibility to injury from subsequent hyperthermy. This "ischemic" effect seems to occur in tumors treated in vivo by immersing the host mice in water at 42-43° C for 15-30 minutes. While such mice were not cured of their cancer the tumors suffered significant metabolic damage to respiration and glycolysis. The injury to metabolism increased over a period of several hours post-hyperthermy. The results indicate that the previously reported greater susceptibility of certain solid tumors to hyperthermic injury in vivo than in vitro, may be due in part to temperature-induced hemorrhage or ischemia of blood vessels supplying the tumor. It is important to distinguish between the direct effects of temperature on cancer and host cells per se and the predisposing effects of temperature-induced vascular failure in the tumor with attendant starvation for glucose and oxygen. The latter may in some cases be deceptive in estimating the thermal exposures required to eliminate metastatic or newly vascularized tumor cells where the oxygen and glucose supplies may not depend on fragile blood vessels such as may occur in large tumors. The ultimate hope is to discover agents and procedures which will bring about a selective sensitization of the cancer cells to heat (whether organized as solid tumors, as freely circulating cells, or as new metastatic foci). Discovery of such agents should be facilitated by in vitro assays of cell metabolism, dye tests

for viability, and transplantation tests together with in vivo tumor growth measurements.

Most recently we have begun investigating the effects of high levels of certain relatively non-toxic vitamins alone and in conjunction with potentiating agents (e.g., amino-triazole, Cu^{++}) at normal (37°) and hyperthermic (43°±) temperatures. One such system currently under study is based on the discovery that riboflavin (vitamin B_2) at 20 micrograms per cc markedly increases the tumor cell damaging effects of copper (3.5 micrograms of Cu^{++}/cc.). The effect is greater under anaerobic conditions than aerobically. It is hoped that such a system may take advantage of the more anaerobic conditions in most tumors as compared to normal tissues. The influence of steroid regulated glucolysis on these interactions with riboflavin and copper will also be studied.

In collaboration with Dr. Katherine Sanford we have further studied the glycolytic properties of mouse tissue culture cell lines derived originally from one normal cell ca 8 years ago. Cells cultured on medium 135 plus 10% horse serum have maintained their original glycolytic characteristics to a remarkable degree. Thus, an original "high" malignancy cell line still has a high glycolysis that is relatively insensitive to inhibition by the anti-insulin Podophyllum fraction, CC7E. In vivo this high line (2472) is characterized by a high percentage of "takes" on transplantation into non-irradiated mice and a short latent period following transplantation. An originally "low" line (2445) is still characterized by a low rate of anaerobic glycolysis that is highly susceptible to anti-insulin inhibition, has a low percentage of "takes" following transplantation to non-irradiated mice, and a much longer latent period. An "intermediate" cell line (2555) is intermediate with respect to these parameters. Thus, there is a good correlation between anaerobic glycolytic capacity (in terms of rate and susceptibility to inhibition by anti-insulins) and in vivo growth characteristics. However, in derivatives of the same three cell lines maintained for a long period on chemically-defined medium (no serum) these distinctions were less clear.

When mouse or hamster cell lines are cultured on medium 135 either with 10% horse serum (HS) or 10% foetal calf serum (FCS), "spontaneous" malignant conversion occurs much more rapidly in the HS medium than in FCS medium (Evans and Sanford). Metabolic measurements of these lines showed that in cells maintained in the presence of HS the glycolytic capacities were greater (higher Q values and reduced susceptibility to anti-insulin regulation) than in cells grown in the presence of FCS. Switching media (HS vs FCS) only 48 hours before glycolytic tests were run, did not reverse the pattern of response in either cell line. Thus, these serum-related differences in glycolysis appear to be long-term effects involving rates of dedifferentiation of the anti-insulin glycolytic control mechanism (hastened by HS and retarded by FCS). Experiments will be carried out to test this hypothesis further.

Honors and Awards: None

Publications:

1. Burk, D., and Woods, M.: Hyperthermy-chemotherapy of mouse melanoma and Ehrlich ascites cancer cells. Proc. 5th Internat. Congress of Chemotherapy (Vienna), pp. 259-262, 1967.

2. Burk, D., Woods, M., and Hunter, J.: On the significance of glucolysis for cancer growth, with special reference to Morris rat hepatomas. J. Nat. Cancer Institute 38: 839-864, 1967.

3. Woods, M., Burk, D., and Hunter, J.: Factors affecting anaerobic glycolysis in mouse and rat liver and in Morris rat hepatomas. J. Nat. Cancer Inst. in press, 1968.

4. Hunter, J., Burk, D., and Woods, M.: Influence of diphosphopyridine nucleotide (DPN) on photodynamic effects of low concentrations of methylene blue in ascites tumor cells. J. Nat. Cancer Inst. 39: 587-594, 1967.

5. Sanford, K., Barker, B., Woods, M., Parshad, R., and Law, L.: Search for "indicators" of neoplastic conversion in vitro. J. Nat. Cancer Inst. 39: 705-734, 1967.

Serial No. NCI-380
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Deoxyribose containing compounds in normal, regenerating and cancer tissues.

Previous Serial Number: Same

Principal Investigator: Walter C. Schneider

Other Investigators: Edward L. Kuff and Emma Shelton, Biosynthesis Section

Cooperating Units: Biosynthesis Section, Laboratory of Biochemistry, NCI

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: The objectives of this project are (1) to isolate and identify deoxyribose containing compounds other than DNA from normal and tumor tissues, (2) to determine the metabolic sequences in which these compounds are involved and whether these sequences are the same in normal and cancer tissues, (3) to isolate DNA from subcellular structures other than nuclei, (4) to study the physical and biochemical properties of extranuclear DNA and (5) to determine the relationship between nuclear and extranuclear DNA in carcinogenesis.

Methods: Nuclei, mitochondria and microsomes were isolated from rat or mouse liver by differential and/or density gradient centrifugation. Nucleic acids were extracted from tissue fractions using sodium lauryl sulfate and phenol. DNA was purified from these extracts by treatment with ribonuclease and passage through Biogel P-60 or by banding in CsCl.

DNA samples were examined by means of analytical CsCl density gradient centrifugation, melting behavior, and amount of labeled thymidine incorporated. DNA was measured by the Burton modification of the diphenylamine reaction.

Major Findings: The research work during the past year dealt entirely with the occurrence of small amounts of DNA in a new subcellular component, the microsomes or endoplasmic reticulum. This was an entirely unexpected finding and considerable effort has been expended in studying this phenomenon. It has been found that the incorporation of thymidine into microsomal DNA was intermediate in extent between that of nuclear and mitochondrial DNA in both

rat and mouse liver. The first reaction to this result was that the microsomal DNA was simply a mixture of nuclear and mitochondrial DNA. The mitochondrial contamination of the microsome fraction could readily be measured using a marker enzyme and demonstrated to be less than 2 per cent. Calbulation of the specific activity of the microsomal DNA using this value showed that it was much lower than the observed specific activity.

The next possibility that was considered was that the microsomal DNA represented a high specific activity type of nuclear DNA that leaked out during the preparation of the microsomes. Although it was possible to demonstrate leakage of DNA from liver nuclei prepared by another procedure the specific activity of this DNA was considerably lower than that of the microsomal DNA. The latter also differed from nuclear DNA in other respects such as difficulty of extraction and insensitivity to deoxyribonuclease. In these respects, the microsomal DNA resembled mitochondrial DNA.

Examination of DNA purified from microsomes, however, showed that it resembled nuclear DNA in every other respect. Both DNA samples had the same buoyant density in CsCl, sedimentation rate and melting temperature. Microsomal and nuclear DNA were both linear in form when examined with the electron microscope and both contained satellite DNA bands when isolated from mouse liver.

To test whether microsomal DNA had a functional significance, microsomes were tested for RNA polymerase activity. It was found that microsomes possessed this activity to about the same degree as nuclei, but a requirement for DNA in the reaction could not be demonstrated with microsomes. This was not surprising, however, since the relative inaccessibility of microsomal DNA had already been noted.

Since the microsomal fraction is known to be hetereogenous cytologically, other experiments were performed to determine the localization of the microsomal DNA. These studies indicated that the DNA was associated with the membranous portions of the microsomes.

Significance to biochemical and cancer research: The finding of DNA in microsomes coupled with previous findings of DNA in melanin granules and mitochondria leads one to speculate that genetic information may be much more widespread in the cell than hitherto imagined. The functional nature of these cytoplasmic DNA's remains to be established, of course.

Proposed course of research: The work on microsomal DNA will be continued. Studies on the turnover of this DNA and on its hybridizability with nuclear and mitochondrial DNA may help to establish more definitely whether microsomal DNA is of nuclear origin. Studies on other organisms which can be ennucleated would also be helpful in this regard. Studies on the effect of hepatocarcinogens on microsomal DNA are also planned.

It is hoped that the experiments on melanin granules can also be completed.

Honors and Awards:

Invited to address pleanary session of Third International Congress of Histochemistry and Cytochemistry in New York, August 1968.

Publications:

1. Schneider, W. C.: Enzymatic Activities of Subcellular Fractions. In Handbook of Biochemistry, H. A. Sober, (Ed.) ppk 3-13, 1968.

Serial No. NCI-373
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on structure and function of nucleic acids.

Previous Serial Number: NCI-373 and 374

Principal Investigator: George W. Rushizky

Other Investigators: Herbert A. Sober, Arnold W. Pratt, Jane N. Toal, and Antoinette E. Greco

Cooperating Units: Dr. W. W. Miller, Department of Chemistry, Pennsylvania State University, University Park, Pa.

Man Years:

Total	: 1.
Professional	: 1/2
Other	: 1/2

Project Description:

Methods Employed: RNA of the E. coli phage MS 2, and DNA of calf thymus were adsorbed on DEAE-cellulose and DEAE-Sephadex. Oligomers of size 10-250 were obtained as described in the last annual report and were characterized by chromatography on DEAE-Sephadex, BioGel P-300, Sephadex G-200, as well as by base ratios, chain length determination with alkaline phosphatase, and by sucrose density gradient centrifugation. In other experiments, polynucleotide kinase was purified from the nuclease free A-19 strain of E. coli, and a cyclic phosphodiesterase from Proteus mirabilis.

Results: Oligomers of DNA obtained by the above procedure corresponded in average chain length to fractions analogously prepared from RNA. Base ratio variations between the oligomer fractions and the original DNA were also found. However, these base ratio variations indicate not a restricted specificity of enzymatic action, but rather a preferential binding of purine-oligomers to the adsorbent DEAE-cellulose, in agreement

with the observations of others. A paper to this effect was published. Oligomers of RNA were used for computer-assisted spectral analyses (see Toal, J. N., et al., Anal. Biochem. May, 1968).

Polynucleotide kinase transfers ^{32}P from ATP^{32} to the 5'OH termini of oligomers. In our hands, this enzyme is contaminated with RNase when prepared as described by others (Takanami et al. J. Mol. Biol. 23: 135 (1967)), even when isolated from the A-19 "RNase-free" strain of E. coli. Furthermore, more than 50-fold molar excess of ATP is needed to label an oligomer. Kinetics of RNA end group labeling with this enzyme are under investigation.

Prior to an investigation of divalent metal catalyzed partial degradation of RNA, the metal content of RNA and DNA has been examined as a function of previous treatment of the RNA, such as EDTA dialysis. This investigation is being carried out with Dr. W. W. Miller of Pennsylvania State, by neutron activation. Preliminary results show that CM-cellulose treated RNA is largely free (less than 2 ppm of Na), of Na^+ and K^+, which would interfere with metal analysis because of their high energy gamma radiation. Neutron activation should give more accurate results than atomic adsorption methods, since for the latter the presence of phosphorus requires muffle furnace temperatures of 700^o or more which may volatilize some metals.

Significance and Proposed Course: Previously, a supply of large oligomers of DNA and RNA was a limiting factor in studies of their fractionation and characterization. Work is now in progress on these oligomers by methods such as end group labeling with polynucleotide kinase and $AT^{32}P$. It is hoped that single large oligomers may be prepared in quantities sufficient for the determination of their nucleotide sequences, which in turn should lead to the nucleotide sequencing of high molecular weight RNA and DNA, not feasible with present methods.

Honors and Awards: None

Publications:

1. Rushizky, G. W., Skavenski, I. H., and Greco, A. E.: Enzymatic hydrolysis of calf thymus DNA adsorbed on DEAE-cellulose. Biochemistry 7: 406, 1968.
2. Rushizky, G. W. and Sober, H. A.: The Preparation and Characterization of Large Oligoribonucleotides. In Progress in Nucleic Acid Research and Molecular Biology, vol. 8, 171, 1968.

Serial No. NCI-381
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Cellular mechanisms in the control of DNA synthesis: Factors influencing thymidylate kinase activity in normal and tumor cells.

Previous Serial Number: Same

Principal Investigator: Ruth K. Kielley

Other Investigator: Remedios M. Avena

Cooperating Units: None

Man Years:

Total:	1-1/4
Professional:	1-1/4
Other:	0

Project Description:

Objectives: The long range objective is to obtain basic information about the behaviour and properties of key enzymes in DNA synthesis in order that we may better understand how cell growth and proliferation may be regulated through activation and repression of their activities. The more immediate objective directed specifically to thymidylate kinase (dTMP kinase) as perhaps the most critical enzyme in triphosphate precursor synthesis, is concerned with enzyme purification, a first and essential requirement for definitive studies on the properties of the enzyme. It is hoped that the information obtained with the purified enzyme will be useful in arriving at an understanding of how this enzyme activity may be altered in the normal and malignant states.

Methods: Enzyme purification methods included the usual techniques of extraction, acid precipitation, ion-exchange chromatography, salt precipitation and gel filtration which were used with the object of obtaining maximum enzyme purity with the highest efficiency. These considerations were of more than usual significance because obtaining the source material,

mouse ascites hepatoma, is a laborious and time-consuming task which does not permit accumulation of large amounts of material; the amount of dTMP kinase to be expected in any tissue is extremely small; furthermore, the necessity to keep the enzyme protected by its substrate, dTMP, at all times throughout the sequence of purification steps adds a cost factor.

The method of purification developed so far to meet these requirements and limitations is as follows: 1) Extraction of enzyme from frozen cells with 0.005 M phosphate buffer, pH 7.4, 2) Precipitation and removal of extraneous protein from the centrifuged extract by acid precipitation at pH 4.8, 3) Passage of neutralized extract through DEAE-cellulose to remove phosphatase activity, 4) Precipitation of the kinase enzyme by $(NH_4)_2SO_4$, between 45 and 65 per cent saturation, followed by dialysis, 5) Gel filtration through Sephadex G-100, 6) Ion-exchange chromatography on DEAE-Sephadex 50A in which the kinase enzyme appears in the effluent, but retarded and separated from the bulk of the protein and 7) Gel filtration through Sephadex G-75 superfine.

dTMP kinase activity was followed by the usual assay method which measures the conversion of ^{14}C-TMP to the di- and triphosphate forms, which after decomposition of unreacted substrate were adsorbed on DEAE Sephadex 25A. After elution, the radioactive products were counted.

The highly purified dTMP kinase finally obtained after gel filtration through Sephadex G-75 superfine represents about a 7000-fold purification over the enzyme in the crude extract, with an overall recovery of about 15 per cent. The amount of enzyme recovered from 500 g of cells was less than 1 mg. In the final gel filtration step, the enzyme corresponded to a distinct protein peak, but showed some overlap with adjacent protein components. Preliminary studies on the purified enzyme indicate that it is strictly a thymidine monophosphate kinase containing less than 0.5% of diphosphokinase. No kinase activity against other nucleoside monophosphates tested could be detected by means of the coupled spectrophotometric assay for ADP. Adenylate kinase was the chief contaminant and the last traces were eliminated only in the last step.

Results and Discussion:

The purification of TMP kinase from mouse hepatoma can be roughly summarized as follows:

Fraction	TMP Kinase Total units *	Specific activity *
Crude extract from 500 g of frozen cells	5000	0.12
Supernatant fraction after pH 4.8 ppt'n.	4000	0.20
After passage through DEAE-cellulose	2800	1.5
$(NH_4)_2SO_4$ ppt. (between 45 and 65% saturation)	2500	1.5
Kinase fraction from Sephadex G-100 filtration	2000	4.5
Kinase fraction from DEAE-Sephadex 50A chromatography	1000	110
Fraction from Sephadex G-75 superfine gel filtration	800	850

*units per mg protein
1 unit equals 1 μmole of TMP phosphorylated per hr at 37°.

Proposed Course of Research: At the present time more enzyme material is being prepared to check the reproducibility of the purification and to secure larger amounts to see if an additional purification step is feasible to reduce adjacent protein components which to some extent overlap into the area of the kinase peak. Present problems in getting sufficient material and processing on a large scale indicate that we may have to settle for the present state of enzyme purity. If so, studies on enzyme properties will have to be made on the enzyme as currently purified.

Significance to Cancer Research: The present work on the purification and characterization of dTMP kinase is part of an attempt to establish a basis for an understanding of the activation and repression of this key enzyme in the regulation of DNA synthesis. Our previous work has shown that a large part of the cellular dTMP kinase of mouse liveris held in an inactive state from which it can be released with resultant activities equal to that of hepatoma. The pertinent question is, "What is the cellular mechanism for keeping the enzyme in a repressed state?" Conversely, the question can be asked, "What turns the enzyme on, as in virus infection?" Kara and Weil (PNAS, January 1967) have shown that upon polyoma virus infection of contact-inhibited mouse kidney cells, there is an immediate stimulation of the DNA-synthesizing apparatus, notably in the phosphorylating kinase enzymes. This stimulation occurs even when DNA synthesis and capsid protein synthesis are prevented by FUdR and appears at the time an intranuclear antigen can be detected. The coincidence of timing suggests that the activator may indeed by the antigen, the synthesis of which is directed by viral DNA. It is conceivable that the activator derepresses the DNA-synthesizing mechanism by triggering the release of active enzyme(s) from the inactive state.

Honors and Awards: None

Publications: None

NCI Serial No. 397
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies comparing the structure, properties and metabolism of the nucleic acids and nucleoproteins of cultured cells and of tumors derived from them.

Previous Serial Number: SAME

Prinicipal Investigator: Jean Rotherham

Other Investigators: None

Cooperating Units: Tissue Culture Section, Laboratory of Biology, NCI

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: To discover changes in structure, properties or metabolism of the nucleic acids or nucleoproteins of cultured cells which are associated with transformation from pre- to post malignant stages

Methods and Major Findings: 1) Pyrimidine nucleoside requirements of a neoplastic strain (NCTC No. 3681) of C3H mouse embryo cells and 2) some aspects of the role of folic acid and vitamin B_{12} in nucleic acid synthesis in these cells.

A neoplastic murine cell strain, NCTC 3681, in culture in chemically-defined medium for several years, utilized labeled thymidine or deoxycytidine for DNA-thymidine synthesis to a greater extent than it did cytidine, uridine, deoxyuridine or orotic acid. The specific activity ratios of DNA-deoxycytidine to DNA-thymidine for these precursors were: deoxycytidine, 1; cytidine, 5; uridine 10. When the folate content of the medium was increased ten-fold there was a greater utilization of cytidine for both DNA pyrimidines and to a lesser extent an increase in the utilization of deoxycytidine, deoxyuridine and uridine for DNA-thymidine. A cell strain was derived from strain NCTC 3681 by growing these cells for 13 transplant generations in medium containing the ten-fold higher level of folate. There was a marked increase in the extent of utilization of cytidine, uridine and deoxyuridine for DNA-thymidine by the derived cells and the specific activity ratios of DNA-deoxycytidine to DNA-thymidine decreased: deoxycytidine 0.3, cytidine 1, uridine 1. The

extent of utilization of orotic acid remained comparatively low.

Data obtained previously in nutritional studies had shown that 3681 cells required vitamin B_{12} for continued proliferation. The vitamin could be replaced by deoxyadenosine and thymidine. When the cells were cultured under these conditions with omission of the vitamin, the effect of addition of the vitamin on the utilization of certain precursors for nucleic acid synthesis could be studied. Thus far little data are available from this study but the data show promise of yielding some significant, more detailed information concerning the involvement of vitamin B_{12} in nucleic acid synthesis in this neoplastic cell strain.

A study was made of the incorporation of precursors into the nucleic acids of NCTC 4823 cells, a non-neoplastic murine cell strain. The methods used were similar to those in the study described above for the neoplastic strain NCTC 3681. The data are incomplete for the 4823 cells but some apparent differences were seen in the extent of utilization of some of the precursors by the two cell strains.

Significance: The studies described have contributed to our knowledge of certain aspects of the synthesis of nucleic acids in a neoplastic cell strain.

Proposed course of work: We propose to obtain more complete data 1) concerning the function of vitamin B_{12} in nucleic acid synthesis in cultured mammalian cells and 2) comparing the metabolism of nucleic acids and nucleoproteins of neoplastic and non-neoplastic cells.

Honors and Awards: None

Publications:

1. Price, Floyd M., Rotherham, J., and Evans, Virginia J.: Pyrimidine nucleoside requirements of a neoplastic C3H mouse cell strain. J. Nat. Cancer Inst. 39: 529 (1967).

Serial No. NCI-399
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Hybridization of DNA and RNA from rat liver mitochondria.

Previous Serial Number: Same

Principal Investigator: Stephen A. Lerner

Other Investigators: Walter C. Schneider

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: The major objective of this project is to determine to what extent DNA and RNA isolated from rat liver mitochondria are able to hybridize with each other. In the larger context, this work is directed toward the understanding of the biosynthesis of mitochondria and its control, and more specifically, what role is played by the accompanying DNA.

Methods: Subcellular components such as nuclei and mitochondria are isolated from rat liver homogenates by differential centrifugation, and the nuclei are further purified by treatment with Triton X-100. Yield of mitochondria is monitored by succinic dehydrogenase assays, and freedom of mitochondria from microsomal contamination is judged by glucose-6-phosphatase assays.

In order to prepare DNA, mitochondria or nuclei are suspended in buffer containing EDTA, to which are added sodium dodecyl sulfate and Pronase. This mixture is incubated at room temperature or 37°C. to effect lysis and protein digestion. The nucleic acids are extracted with phenol, and following several precipitations with ethanol to remove the phenol, the nucleic acids are dissolved in buffer containing EDTA and then incubated

with pancreatic and T_1 ribonucleases. The digest is then incubated with Pronase, followed by phenol extraction and ethanol precipitations. Residual RNA digestion products and phenol are removed by passage of the mixture over a column of Sephadex G-50.

In order to prepare RNA, mitochondria or nuclei are suspended in buffer containing magnesium, to which is added sodium dodecyl sulfate. Scrupulous attention is paid to keeping the preparations at 0-4°C. or as near as possible at all times, lest endogenous ribonucleases digest the RNA's and render them unrecognizable by size criteria. Repeated phenol extractions are then carried out. Following ethanol precipitation, the nucleic acids are redissolved in buffer containing magnesium, and incubated sequentially with deoxyribonuclease and then Pronase. The preparations are then subjected to a series of phenol extractions and ethanol precipitations. The various species of RNA are separated by centrifugation on a preparative scale through linear sucrose density gradients. Fractions are pooled and passed through a column of Sephadex. RNA's are prepared similarly from post-mitochondrial supernatant fluid.

Hybridization is carried out according to a procedure of Gillespie and Spiegelman, in which denatured DNA adheres to nitrocellulose membranes, and labeled RNA which hybridizes with the adherent DNA then remains on the membranes where it can be counted.

Results: The foregoing procedures for preparing DNA's and RNA's have been worked out to obtain DNA free from RNA and vice versa. It had been hoped that this could be accomplished without the use of nuclease digestion of the unwanted nucleic acid, but the uncertainties of nucleases and the concern over their subsequent removal were overshadowed by the ease of preparation when they are used. Furthermore, it was found that the use of agarose columns, which had been used to separate undigested DNA and RNA, introduced difficulties because of the long time periods for elution, measured in days. Although these columns were kept in the cold room, there was noticeable degradation of RNA, probably due to residual endogenous ribonuclease or exogenous ribonuclease, perhaps from bacteria, which were found to have colonized the column despite the inclusion of 0.5% butanol as a bacteriostatic agent.

Other major changes in procedure included substitution of ethanol precipitations for dialysis in removing phenol and digestion products (to obviate the need for long periods at cold room temperature), and storage of nucleic acid preparations in the freezer after freezing in dry ice-ethanol rather than storage in the cold room.

Separation of the various species of RNA's was carried out on sucrose density gradients with fair to good resolution of peaks. Major peaks were found at approximately 4, 8, and 28 S from nuclear, mitochondrial, and cytoplasmic preparations. Mitochondrial preparations had variable amounts of minor peaks, one in the valley between 4 and 18 S peaks, and two on the heavy side of the 18 S peak. Ribonuclease activity present in the preparations was assayed by incubation of the preparation in question at a given temperature and for a given period of time, and sucrose gradient patterns from before and after incubation were compared. Degradation of RNA could be recognized by diminution of 18 and 28 S peaks and a concomitant rise in the 4 S and minor peaks. Eventually mitochondrial RNA preparation could be obtained which exhibited little alteration of peaks after incubation at 25°C. for 24 hours.

Labeling of RNA's was accomplished by intraperitoneal injection of 3 mc of 5-H^3-orotic acid in divided doses over 24 hours into each rat. Specific activities on the order of 1 million cpm per micromole of RNA-phosphorous were obtained.

Significance to Biochemical Research:

The previous findings of DNA in mitochondria of various tissues from organisms of diverse phyla suggest a possible reservoir of heritable information outside the nucleus, which might be operative in the phenomena of extranuclear inheritance. The likely homology of mitochondria to chloroplasts, which have been recognized for some time to contain DNA, and the suggested possible relationship of mitochondria to autonomous organisms such as bacteria provide impetus for the investigation of possible autonomy for mitochondrial biosynthesis which endogenous DNA might afford. If mitochondrial RNA is found to hybridize with the DNA, this would suggest a transfer of information from the DNA, perhaps analogous to the RNA-mediated expression of information in protein synthesis, as reported in micro-organisms.

Proposed Course of Research:

It is planned to test hybridization between unlabeled nuclear and mitochondrial DNA's and the various labeled nuclear, mitochondrial, and cytoplasmic RNA's. Unlabeled RNA's are being prepared for use in competition experiments.

Honors and Awards: None

Publications: None

Serial No. NCI-5205
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on biochemical mechanisms of sensitivity and resistance to antimetabolites.

Previous Serial No.: Same

Principal Investigator: Elizabeth P. Anderson

Other Investigators: B. Winfred Ruffner, Jr., and Joseph E. Ciardi

Cooperating Units: None

Man Years:

Total:	3
Professional:	3
Other:	0

Project Description:

A. Studies on glutamine antagonists.

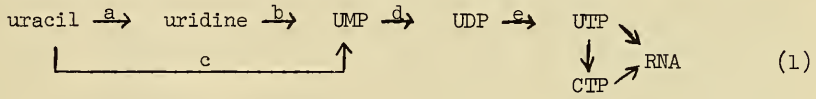
This problem has been concerned with biochemical aspects of resistance to the glutamine antagonists azaserine, diazo-oxo-norleucine, and duazomycin A, in sublines of the mouse plasma cell neoplasm 70429. Work on this project in the past year has been concerned only with extending and summarizing our evaluations of the previous five years of work in this area, and has not included additional experimentation. The completed work now being summarized was concerned with characterizing, biochemically, the extent of inhibition by the analogs at four different sites of action and comparing, in the sensitive and resistant lines, this extent of inhibition, and the speed and completeness of the recovery from it. Differences between the sensitive and resistant lines, as judged by these parameters, have been found at all four sites of action and have been quantitated.

This work has involved the isolation and identification of various purine and pyrimidine derivatives that were metabolites, both anabolic and catabolic, of the radioactive precursers used in these studies. Some of these separations and identifications have used the relatively new procedures of chromatography on thin-layer cellulose. A manuscript describing the use of thin-layer chromatography for this work has been written by J. E. Ciardi and E. P. Anderson, entitled, "Separation of Purine and Pyrimidine Derivatives by Thin-Layer Chromatography". This was submitted and accepted for publication this year and is forthcoming in Analytical Biochemistry.

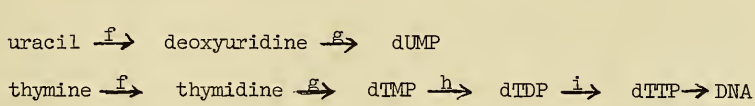
Proposed Course of Project: It is proposed to extend this study, experimentally, in the direction of exploring and quantitating glutamine synthetase in the lines sensitive and resistant to the glutamine antagonists, and examining the response of this enzyme to the antagonists in both cell lines. Since differences exist between the sensitive and resistant lines at all four sites of antagonist action studied so far, and since these sites represent four different enzyme proteins, it would seem that, in seeking an explanation for this breadth of action, some common denominator such as glutamine synthetase should be explored.

B. Studies on pyrimidine anabolism.

This continues our previous work on pathways of biosynthesis of pyrimidine nucleotides, mechanisms of control over these pathways, and anabolism os pyrimidine analogs. Most of our present studies in this area are concerned with "salvage" pathways of pyrimidine nucleotide biosynthesis:



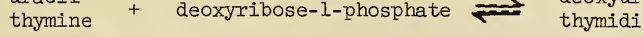
(1)



(2)

1. Studies on deoxyuridine (thymidine) phosphorylase

A manuscript has been drafted by G. M. Lyon, Jr. and E. P. Anderson, entitled, "Deoxyuridine Phosphorylase from Tetrahymena Pyriformis", and covering the study done with Dr. Lyon (164-66) on deoxyuridine (thymidine) phosphorylase. This enzyme catalyzes the reversible phosphorylation of pyrimidine deoxyribonucleosides (reaction f above):



(3)

This study has used the pyrimidine-requiring protozoan, Tetrahymena pyriformis, which does not synthesize the pyrimidine ring de novo but which can grow on uracil or its nucleoside; it therefore makes pyrimidine nucleotides from the free base by the "salvage" pathways. The enzyme has been purified 60-fold from frozen-thawed lysates of Tetrahymena; the most highly purified fractions contained only four protein bands in disc electrophoresis on polyacrylamide gel, and the $s_{20,w}$ was approximately 5.5 in density gradient centrifugation. The kinetic data on both the synthetic and degradative reactions of the enzyme suggest that the enzyme binds uracil in two distinct ways, one catalytic, a second non-catalytic and inhibitory. The work on this problem has been extended in the past year in more mathematical evaluation of the kinetic data with regard to their significance for the binding sites for uracil and the

mechanism of these two bindings. This evaluation has been made with the kind assistance of Dr. John Mullooly, Biometrics Research Branch, National Heart Institute.

2. Studies on uridine anabolism. Uridine kinase and uridine phosphorylase in tumor sublines sensitive and resistant to pyrimidine analogs.

We have previously reported our findings on decreased activity of uridine kinase (reaction b above) associated with resistance to fluorouracil or fluorouridine in the mast cell neoplasm P815. In these tumor sublines (P815/FU and P815/FUR) studies on biological resistance, tracer studies in vivo, and enzyme studies in vitro have all, consistently, indicated a decreased capacity for anabolism of pyrimidines and pyrimidine analogs. In the enzyme studies, anabolism of uridine in the resistant lines was 25-50% of that in the sensitive line. For this work uridine kinase has been assayed as anabolism of uridine-C^{14} to total nucleotides. Characterization of this enzyme activity has been pursued further by Mr. Ciardi. K_m values for the substrate uridine were very similar in the sensitive and in both resistant lines. K_m values for the cosubstrate ATP, in the presence of an ATP-regenerating system, were also found to be similar in the sensitive and resistant sublines; this K_m for ATP was 2.5-fold greater than that for uridine. Studies with both substrates showed V_{max} values to be lower in the preparations from the resistant lines. The metal ion requirement was likewise similar for all tumor lines studied. Effects of anions have also been studied; phosphate was found to stimulate enzyme activity, to a somewhat greater extent in the P815/FUR line; this anion stabilized activity in preparations from both sensitive and resistant lines. Sulfate ions were found to inhibit the reaction, and this could be reversed by phosphate.

Additional experiments have been carried out to explore inhibition of enzyme activity in the presence of high concentrations of ATP, which was especially pronounced in the absence of an ATP-regenerating system. The results supported the conclusion that ADP was a more potent inhibitor than ATP but that inhibition might be exerted by both ATP and ADP. A similar situation was noted with GTP and GDP; unlike ATP, GTP did not serve as phosphate donor for the reaction. Inhibition by these purine nucleotides might serve as a control mechanism over pyrimidine nucleotide biosynthesis, so that interaction between purine and pyrimidine derivatives could help to keep the pool of polynucleotide precursors balanced with respect to each other. Of several purine and pyrimidine nucleoside triphosphates tested, only dATP could partially substitute for ATP as phosphate donor for uridine kinase. Feedback inhibition by CTP and UTP, which was found in all types of enzyme preparations studied, was also observed through several steps of purification of the enzyme. Susceptibility to feedback inhibition was found in preparations from both the sensitive and resistant tumor lines, but was less pronounced in the latter case.

The further anabolism of UMP in this system was also explored by quantitating the accumulation of radioactivity from C^{14}-uridine in the separate mono-, di-, and triphosphates of uridine. A time study has shown that, over a 60-minute period, there was greatest accumulation of UTP, one-third as much of

UDP, and essentially no UMP. Studies on the effect of ATP concentration showed that at low ATP levels UMP accumulation was greatest, whereas at optimum ATP accumulation of labeled UMP was negligible and the radioactivity accumulated almost entirely in UTP. This suggested a higher affinity of uridine kinase for ATP and supported our previous conclusion that uridine kinase was the rate-limiting reaction. PCMB was found to be a more potent inhibitor of UMP and UDP kinases than of uridine kinase; thiouracil also appeared to inhibit the two higher kinase reactions. Studies on uracil metabolism have also been carried out. Surprisingly, nucleotides that inhibited uridine anabolism also inhibited its catabolism as assayed under conditions optimum for kinase activity; these effects on uridine cleavage cannot be clearly ascribed to effects on uridine phosphoyrlase, since these same nucleotides produced different effects on uridine phosphorylase measured as reaction of uracil with ribose-1-phosphate.

Partial purification of uridine kinase has been carried out in parallel from frozen-thawed lysates of P815/S and P815/FUR, using high-speed centrifugation, precipitation at pH 5, ammonium sulfate fractionation, dialysis, and gel filtration on Sephadex. This was done to explore differences between the kinases from sensitive ("wild type") and resistant (mutant) cells, and suggestive differences have been found. These were primarily differences in protein stability, the enzyme from P815/FUR being, for example, more labile to centrifugation at 100,000 x g and to acid precipitation. Also, with partially purified preparations of the P815/FUR enzyme, the reaction velocity increased with protein concentration at a non-linear rate, the plots exhibiting a curvature upward; in contrast, the same plots for P815/S fractions were linear. Such data would be obtained if there were an associable activator present in the P815/FUR enzyme preparations such that more of the activated enzyme existed at high protein concentrations. This activation could be due to association of the enzyme with a small, non-protein cofacter, or to association of inactive enzyme subunits into an active enzyme complex. The lability of the P815/FUR enzyme has made it difficult to select purification steps good for both preparations, and the results to date serve to indicate differences in the enzymes but do not represent optimum purification.

The studies on uridine kinase have been extended to lines of the plasma cell neoplasm 70429 as well, and the enzyme protein appeared to be similar to that in the 815 lines. Careful studies have now shown that in azauracil-resistant line of this tumor (70429/AzaU), resistance can also be correlated with a decrease in uridine anabolism (essentially uridine kinase activity). This decrease was smaller than that in the fluoropyrimidine-resistant 815 lines, only about 20% relative to the level in the sensitive line preparations, but it seemed to be consistent and is now considered to be significant. No such decrease in uridine kinase activity could be detected, however, in our enzyme preparations from 70429/AzaUR, selected for resistance to azauridine, and clearly resistant, biologically, to both azauracil and azauridine. It appears that some other mechanism of resistance must be involved in this mutant line.

The studies on this problem this year have been pursued by Mr. Ciardi,

and these results have been written up in his thesis for the Ph.D. degree in biochemistry, entitled, "Pyrimidine Nucleotide Biosynthesis in Neoplastic Cells: the 'Salvage' Pathway via Uridine Kinase".

Proposed Course of Project: Work is now in progress to purify further, in parallel, the kinase activities from preparations of P815/S and P815/FUR, and to study similarities and differences between the two enzymes; it may be possible, in this way, to distinguish between a lower concentration of kinase in the resistant cells and the presence of a kinase with decreased specific activity. Two manuscripts are planned covering the work already completed or in progress, one on characterization of the kinase in the various tumor lines and its decrease in analog-resistant lines, and another on the purification studies.

3. Studies on UMP kinase.

Following up our previous study on deoxyuridine phosphoylase in Tetrahymena pyriformis (B. 1. above) further work has been carried out by Dr. B. W. Ruffner on pyrimidine metabolism in this organism. This protozoan is unable to synthesize the pyrimidine ring de novo, and is dependent on "salvage" pathways for pyrimidine anabolism; either cytidine or uracil must be present in the culture medium for the organism to synthesize DNA or RNA. The presence of a potent cytidine deaminase suggests further that uracil and UMP are probably common intermediates for all pyrimidine ribonucleotide synthesis in these cells. Our preliminary studies have indicated that uridine kinase is very low in Tetrahymena pyriformis, in contrast to the results with mammalian cells. Correlative studies in Dr. R. W. Brockman's laboratory have indicated that the major salvage pathway used by this organism for ribonucleotide synthesis may be, not the phosphorylase-kinase pathway (reactions a, b in Scheme 1 above), but rather an active PRPP-UMP phosphoribosyl transferase whereby uracil is converted directly to UMP (reaction c in Scheme 1). We have therefore undertaken a study of the further anabolism of UMP in Tetrahymena, especially of of UMP kinase (reaction d) and of possible mechanisms of control over anabolism exerted at this step. This reaction has been assayed as phosphorylation of labeled UMP.

It has been possible to purify the enzyme 300-fold, with 30% recovery, from frozen-thawed lysates of Tetrahymena, using high-speed centrifugation (100,000 g for 90'), precipitation at pH 5, ammonium sulfate fractionation, and gel filtration on Sephadex. In the purified preparation all nucleoside diphosphokinase activity (measured as phosphorylation of labeled UDP) was found to have been removed, and ATP consumption was stoichiometric with UDP synthesis. CMP kinase activity paralleled UMP kinase through all purification steps. This preparation can be stored at 4° C with good stability; less of enzyme activity is approximately 20% over a two-week period. Further purification steps have been tried, but to date have resulted in unstable preparations, apparently because of protein dilution. A three-fold dilution of the preparation of Sephadex could, alone, produce rapid loss of activity; this effect could be delayed by the addition of bovine serum albumin, reducing agents (mercaptoethanel or dithiothreitol), or ATP.

All naturally occurring ribo- and deoxyribonucleoside di- and triphosphates have been tested as inhibitors of this enzyme. The following nucleotides inhibited UMP kinase in the presence of saturating UMP: CDP > APD > dADP > CTP > UDP. At one-tenth substrate concentration, CDP inhibited 60% and UDP 10%. Kinetic studies suggested that ADP inhibition was competitive with ATP, and UDP was competitive with UMP. CDP appeared to increase the affinity of UMP binding, but to decrease markedly the maximum velocity. In this preparation kinase activity with CMP as substrate was inhibited only minimally by CDP and UDP. The magnitude of ADP and dADP inhibition was similar to that observed against UMP kinase activity, but Dixon plots (1/v against I) at two concentrations of ATP suggest that the K_i's for ADP are different for the two activities in this enzyme preparation; 1.75×10^{-3} with UMP as substrate, and 0.9×10^{-3} with CMP as substrate. The observed parallel purification of the two activities suggests that UMP and CMP are being phosphorylated by the same enzyme, but the differences in inhibition of the two activities by UDP, CDP, and ADP weigh, perhaps, against this conclusion. The most likely possibilities are that two very similar enzyme proteins have been purified in parallel through the steps used to date, or that a single enzyme protein mediates both phosphoyrlations but has separate, though interacting, sites for binding UMP and CMP.

Proposed Course of Project: Present efforts are directed toward further purification of the enzyme(s) and more detailed studies on the characterization and kinetic aspects of the observed inhibition.

Honors and Awards: None

Publications:

1. Ciardi, J. E. and Anderson, E. P.: Separation of purine and pyrimidine derivatives by thin-layer chromatography. Anal. Biochem. 22: 398, 1968.

2. Ciardi, J. E.: Pyrimidine nucleotide biosynthesis in neoplastic cells: The "salvage" pathway via uridine kinase. Ph.D. thesis (Biochemistry), George Washington University, April 1968.

Serial No. NCI-5207
1. Laboratory of Biochemistry
2. Nucleic Acids Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Purification of Phosphorylcholine-Cytidyl Transferase.

Previous Serial Number: NONE

Principal Investigator: W. Guy Fiscus

Other Investigators: W. C. Schneider and S. R. Himmelhoch

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: The objectives of this project are to 1) purify and 2)characterize the enzyme Pc-Cytidyl transferase. Interest in this particular enzyme stems from certain observations made in this laboratory: 1) a product, dCDP-choline, is present in liver tumor tissue in much higher concentration than in normal liver, 2) the enzyme is activated by altered phospholipids in a pattern suggesting a regulatory role in phospholipid metabolism.

Methods: Purification procedures that have been applied are 1)centrifugation to obtain soluble proteins 2) precipitation techniques utilizing acetone, butanol and ammonium sulfate, and 3) chromatography using ion exchange and gel filtration methods.

Findings: Partial purification of this enzyme has been accomplished resulting in 45-fold increase in specific activity with good recovery at total activity. However, it has not yet been possible to either obtain high levels of purification or to study the properties of the partly purified state due to instability of activity.

An inhibitor of this enzyme, possibly a polyribonucleotide, has been found in rat liver.

Proposed course of research: Attempts are being made to stabilize the enzyme with initial success having been demonstrated using the thiol compound, mercaptoethanol. A scheme of partial purification will be worked out and the properties of the purified enzyme will be studied. Finally, isolation and

identification of the inhibitor can be attempted.

Honors and Awards: None

Publications: None

Serial No. NCI-306
1. Laboratory of Biochemistry
2. Nutrition and Carcinogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on β-hydroxy-α-amino acids.

Previous Serial Number: Same

Principal Investigator: Theodore T. Otani

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/3
Professional:	1/3
Other:	0

Project Description:

Objectives: Stemming from a report by Levi and Blondal that sodium N-chloroacetyl-DL-serinate caused a complete regression of Sarcoma 30, a series of "unnatural" β-hydroxyamino acids prepared in this laboratory was tested for anti-tumor action using microbial and animal anti-tumor screening systems. Both the free amino acids and their corresponding N-chloroacetyl derivatives were tested. Certain of these were shown to possess some biological action in terms of microbial growth inhibitory capacity and exhibited moderate tumor growth inhibitory action. The objective of this project is to determine whether or not any of these "unnatural" β-hydroxyamino acids and/or their derivatives will be sufficiently tumor inhibitory so as to be chemotherapeutically useful. Any tumor inhibitory action noted will furnish useful information in the designing of more effective anti-tumor agents. These β-hydroxyamino acids as chemically prepared are racemic mixtures of the diastereomers. Earlier microbial and animal anti-tumor screens showing modest activity have been performed using the mixture of the four isomers. The effect observed was, therefore, that of the active agent diluted with the other three isomers.

The four isomers of β-hydroxynorleucine and the chloroacetyl derivatives of each have been isolated in sufficient quantities for microbiological anti-tumor screens.

When the chloroacetyl derivatives of each of the four isomers of β-hydroxynorleucine were tested for anti-tumor activity utilizing Lactobacillus casei 7460, it was found that the D-isomers of both diastereomers A and B were growth inhibitory (to the extent of about 35 percent), and the L-isomers were either growth stimulatory or inactive. These findings indicated that the laborious separation of the diastereomers was unnecessary, and that only a removal of the L-isomers by enzymatic hydrolysis of the diastereomeric antipodes was called for, yielding N-chloroacetyl-D-β-hydroxynorleucine AB.

It has been known for some time that the N-trifluoroacetyl derivatives of straight chain amino acids were hydrolyzed irrespective of the optical configuration of the amino acid. An assumption was made that the efficacy of the N-acyl-β-hydroxyamino acid as growth inhibitory agent is due, first, to its ability to penetrate the cell membrane as an uncharged molecule, and, second, to the facility with which it is hydrolyzed within the cell and thus releasing the unnatural amino acid in the immediate proximity of incorporation for protein synthesis. When a racemic mixture of an amino acid is used, it is reasonable to expect enhanced efficacy for a given molarity of substrate if both antipodes were susceptible to hydrolysis. Such was the rationale in preparing the trifluoroacetyl derivatives of β-hydroxynorleucine.

N-Trifluoroacetyl-DL-β-hydroxynorleucine AB was prepared under the conventional Schotten-Baumann conditions. Products with two ranges of melting points were obtained: m. p. 174-175, and m. p. 212-224°C. As the quantity was limited characterization was not carried out at this time. Rather, a first approximation determination on their growth inhibitory action was made using the microbial screening system (L. casei 7469).

Finding:

1. It was found that both preparations of N-trifluoroacetyl-DL-β-hydroxynorleucine AB when tested for growth inhibitory action against L. casei 7469 showed no inhibitory action, up to a final concentration of 1 mg/ml.

2. As it was found that only the D-antipode of the diastereomers were active and no separation of the diastereomers of β-hydroxynorleucine was required, a large scale production of N-chloroacetyl-D-β-hydroxynorleucine AB has been undertaken. Gram quantities of its DL-β-hydroxynorleucine AB and N-chloroacetyl-DL-β-hydroxynorleucine AB, have been prepared.

Significance: The earlier observation that a β-hydroxyamino acid and its N-chloroacetyl derivative did show some tumor inhibitory activity, both in terms of smaller tumor size, and of increased survival time in certain solid tumors is encouraging in view of the relatively small number of compounds showing such activity. If increased inhibitory effect could be observed by use of the pure active isomer over the activity shown by a racemic mixture of the diastereomers, or by the preparation of a more potent β-hydroxyamino acid, (as suggested by findings of increased susceptibility to certain enzymes or of increased anti-metabolic properties of structurally, closely-related compound, β-hydroxy-methionine, for example) this group of compounds would offer a new class of tumor inhibiting agents.

The finding of increased growth inhibitory activity in some organism by N-chloroacetylation calls for re-examination of many of the amino acid analogs previously shown to be potent antimetabolites but impracticable in cancer chemotherapy because of the nullifying action of the natural analog present in the organism. N-Chloroacetylation, perhaps, may protect the active analog until it reaches the site of high growth activity, for example, the periphery of growing tumors, where peptidase, and hence likely acylases, would be present in higher concentrations. N-Trifluoroacetylation, in addition, may facilitate concentration build-up of the active, free D-β-hydroxyamino acid at this site because of the susceptibility of the trifluoroacetyl derivatives of both antipodes to acylase action. Trifluoroacetyl derivatives of certain class of amino acids (straight chain), unlike the corresponding chloroacetyl derivatives, do not exhibit rigid optical stereospecificity, in that the trifluoroacetyl derivatives of both D- and L-isomers are hydrolyzed by acylase.

Proposed Course of Study:

1. Larger quantities of N-trifluoroacetyl-DL-β-hydroxynorleucine must be synthesized so that characterization of the product could be made. The characterization will involve:

a) Elemental analysis, b) hydrolysis and paper chromatographic identification of the amino acid thus released, c) Van Slyke ninhydrin CO_2 determination to establish the position of the acylation (acylation of the amino or the hydroxyl group).

2. Large scale production of N-chloroacetyl-D-β-hydroxynorleucine AB will be made.

3. The N-Chloroacetyl-D-β-hydroxynorleucine thus prepared will be tested against tumors in mammalian systems (Carcinoma 755, Sarcoma 180, and Leukemia 1210).

4. Further attempts to prepare β-hydroxymethionine will be made.

Honors and Awards: None

Publications: None

Serial No. NCI-394
1. Laboratory of Biochemistry
2. Nutrition and Carcinogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on isozyme distribution in various transplantable rat hepatomas.

Previous Serial Number: Same

Principal Investigators: Theodore T. Otani and Harold P. Morris

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1-2/3
Professional:	1
Other:	2/3

Project Description:

Objectives: In our earlier studies, it was found that aspartate aminotransferase (GOT) of several chemically-induced transplantable rat hepatomas which were developed in this laboratory, like that of normal rat liver, consisted of at least two isozymes. One of these moved as an anion and the other as a cation in an electric field. The corresponding anionic and cationic isozymes of the normal rat liver preparation and of the hepatomas moved identically when subjected to electrophoresis on granular starch. The relative distribution of each isozyme, however, differed among the various hepatomas, and the relative distribution in hepatomas differed from that of normal liver. It was further found that although no marked effect was observed on the specific activities and on the relative isozyme distribution of the GOT from normal rat liver after adrenalectomy, hydrocortisone treatment, or both, there appeared to be some responsiveness by GOT of Hepatoma 5123B to these treatments. Adrenalectomy effected a decrease in specific activity of the GOT from Hepatoma 5123B, while hydrocortisone caused a slight increase in specific activity, as well as a

change in the isozyme distribution in favor of the cationic or mitochondrial component. A combination of adrenalectomy and hydrocoritsone treatment caused a normalization of the two effects on the specific activity, and an alteration of the isozyme distribution favoring the cationic component. A time-course study of the effect of hydrocortisone on the specific activity showed that the increase in activity is discernible within 2 hours after hydrocortisone administration and that the activity reaches a plateau in about 5 hours.

The aim of this project has been to compare the distribution of isozymes of various enzymes in the normal liver and in hepatomas, and to note the effect of various stimuli, such as adrenalectomy and hormonal treatment, on the distribution of these isozymes. The immediate objectives through this period have been to note the differences in isozyme distribution of alanine aminotransferase and L-malate: NAD oxidoreductase in the normal livers and in hepatomas.

Major Findings:

1. Using granular starch block electrophoresis, the existence of at least two isozymes of alanine aminotransferase (GPT) has been confirmed. However, complete separation of these two isozymes has not been accomplished thus far.
2. One isozyme moves slightly cathodic and the other slightly anodic at pH 5.6; the prominent peak is the cathodic component.
3. Although confirmation must be made after the isozymes are separated more distinctly, it appears that the anionic peak is present in significantly less amount, or is absent in the tumor GPT.
4. L-malate: NAD oxidoreductase (MDH) has been resolved into at least four isozymes, one prominent cationic and three anionic components. The anionic components have not yet been separated distinctively.
5. No marked difference has been noted thus far in the relative distribution of the anionic MDH of normal liver and hepatoma but conclusions must await a more distinct separation of the components.

Significance: The enzymes aspartate aminotransferase and alanine aminotransferase hold a key position in the intermediary metabolic pathway of an organism, occupying a pivotal position between amino acid and carbohydrate metabolism. There exists two biochemically distinct GOT and GPT isozymes in rat liver, one primarily situated in the mitochondrial and the other in the supernatant fraction. There exists at least four isozymes of MDH in the normal liver and in the hepatoma, similarly some localized in the mitochondrial and others in the supernatant fraction. It is possible that each isozyme by its location in the cell has a definite function, for example

in the case of GOT, whether to mediate the conversion of α-ketoglutarate to glutamic acid, hence channeling substrates into the amino acid pathway, or to mediate conversion of glutamic acid to α-ketoglutarate, hence channeling substrates to energy production. In the normal cell, there is likely a delicate balance between these systems, perhaps, in some way influenced and controlled by hormones. It has been reported that there are less mitochondria in tumor cells than in the normal liver parenchymal cells. Could one phase of a mechanism initiating neoplasia be due to a disruption of this balance? The findings from these studies, it is hoped, will contribute to the elucidation of this problem.

Proposed Course of Study:

1. Although different conditions have been tried to separate the isozymes of GPT more distinctly, complete separation has not been thus far successful. Further attempts will be made using other supporting media, such as Sepraphore III and starch gel.
2. Similarly, complete separation of the isozymes of MDH will be attempted.
3. Studies will be made on the interconversion of the mitochondrial and the supernatant GOT as well as those of MDH.

Honors and Awards: None

Publications: None

Serial No. NCI-324
1. Laboratory of Biochemistry
2. Nutrition and Carcinogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biological and biochemical studies of the mechanism(s) of carcinogenesis.

Previous Serial Number: Same

Principal Investigator: Harold P. Morris

Other Investigators: Theodore T. Otani and Billie P. Wagner

Cooperating Units: Dr. W. M. Lauter, Department of Pharmacology, University of Florida, Gainesville, Florida; Drs. Van R. Potter and H. Pitot, McArdle Memorial Laboratory, University of Wisconsin, Dr. George Weber, University of Indiana Medical School, Indianapolis, Indiana; Dr. Sidney Weinhouse, Fels Cancer Research Laboratory, Temple University, Philadelphia, Pennsylvania; Dr. P. Ofner, Lemuel Shattuck Hospital, Boston, Massachusetts; Dr. H. Kalant, Department of Biochemistry, University of Toronto, Toronto, Canada; Dr. J. O. Ambrus, Roswell Park, Buffalo, New York; Dr. B. R. Munson, Department of Biochemistry and Pharmacology, State University of New York at Buffalo, New York; Dr. Testsuo Ono, Cancer Institute, Tokyo, Japan; Dr. Takashi Sugimura, Biochemistry Division, National Cancer Center, Research Institute, Tokyo, Japan; Dr. S. Süss, Max-Planck Institute für Virusforchung, Heidelberg, Germany; Dr. Dean Burk and Dr. Mark Woods, Laboratory of Biochemistry, NCI; Dr. S. Abraham, Children's Research Foundation, Oakland, California; Dr. J. C. Elwood, Upstate University, Department of Biochemistry, Syracuse, New York; Dr. A. Schwartz, Department of Pharmacology, Baylor University College of Medicine, Houston, Texas; Dr. J. Drews, Yale University College of Medicine, New Haven, Connecticut; Dr. N. Katunuma, Department of Enzyme Research, Tokushima University School of Medicine, Tokushima, Japan; Dr. H. J. Fallon, Department of Medicine, North Carolina College, Chapel Hill, North Carolina; Dr. I. Seidman, New York University College of Medicine, New York; Dr. M. Bitensky, New York University College of Medicine, New York; Dr. C. Wu, University of Michigan Hospital, Ann Arbor, Michigan; Dr. R. Baserga, Fels Research Institute, Philadelphia, Pennsylvania; Dr. H. Busch, Baylor University College of Medicine, Houston, Texas; Dr. P. Byvoet, University of Florida College of Medicine, Gainesville, Florida; Dr. R. Estabrook, Johnson Foundation, University of Pennsylvania, Philadelphia, Pennsylvania; Dr. H. Coon, Carnegie Institution, Department of Embryology, Baltimore,

Maryland; Dr. H. Elford, University of Michigan College of Medicine, Ann Arbor, Michigan; Dr. W. B. Looney, University of Virginia, College of Medicine, Charlottsville, Virginia; Dr. P. W. Majerus, University of Washington, College of Medicine, St. Louis, Missouri; Dr. H. Mayaji, Wakagama Medical School, Wakagama, Japan; Dr. T. Otani, Laboratory of Biochemistry, NCI; Dr. S. Odashima, Sasaki Institute, Tokyo, Japan; Dr. G. Schreiber, Biochemisches Institute, University Freiburg, Breisgau, Germany; Dr. Harry Brown, Cancer Research Center, Columbia, Missouri; Dr. E. Bresnick, Department of Pharmacology, Baylor University College of Medicine, Houston, Texas; Dr. D. R. Meranze, Philadelphia, Pennsylvania; Dr. D. Neubert, Pharmacological Institute, Free University, Berlin, Germany; Dr. Peter Nowell, University of Pennsylvania, School of Medicine, Department of Pathology, Philadelphia, Pennsylvania; Dr. R. Rosso, Pharmacological Institute, Mario Negri, Milan, Italy; Dr. M. J. Sweeney, Lilly Research Laboratories, Indianapolis, Indiana; Dr. A. Vescia, Fondazione, Senatore Pascale, Naples, Italy; Dr. T. E. Webb, McGill University Cancer Research Unit, Montreal, Canada.

Over 40 cooperating units within and without NIH are active in various aspects of this hepatoma research. A few who were active during 1966 were inactive during 1967, but several new laboratories have become active as indicated above. In addition, the animal laboratories were on a contract with Melpar, Inc., Falls Church, Virginia.

This is a summary report, covering the first fourteen months work, for a program under contract PH-43-66-51, Melpar, Inc., concerned with the production of minimal deviation hepatomas and other rodent malignancies. In the course of this work, emphasis has been placed on the induction of new tumors by chronic feeding of low levels of carcinogens, and on insuring the continued availability of large numbers of rats bearing different lines of minimal deviation hepatomas. Facilities and personnel were provided for the preparation of stock and carcinogen-containing diets, for the maintenance of control and tumor-bearing animals and fixing of tissues, and for the shipment of tumor-bearing animals to collaborative investigators. Dr. David R. Meranze has, on a consultant basis, carried out pathological examinations of selected tumor tissues. A hepatoma tumor bank was started. It is too soon to report on the retention of viability by these hepatoma cells in the bank.

Details of diets, autopsy methods, and transplantation procedures are given. Many new hepatoma lines were started during the year.

Two strains of rats, Buffalo (Buff) and ACI, have been used. Animals already on experiments, as well as stock animals, were obtained from the National Cancer Institute shortly after initiation of this program. During the course of the work, weanling animals have been received from several sources: (a) the breeding colony at the National Institutes of Health, (b) Texas inbred, and (c) Simonsen Laboratories.

Diet

With the exception of rats on carcinogenic diets and their controls, all

animals are maintained on NCI formula pelleted feed (Emory Morse Company, Gilford, Connecticut) fed ad libitum. This formula was developed by the project officer. Three chemicals have been added to basic diet 222A for the purpose of inducing new transplantable tumors. The following chemicals are being used: Aflatoxin B_1; N-fluorenyl-2-nicotinamide; and N-2-fluorenyldiacetamide. Attempts were made to induce additional diploid tumors. Results are not yet available.

All incoming animals are routinely placed in the quarantine room and held for a minimum of two weeks before being transferred to the stock room. Animals for tumor induction, as well as their controls, are taken from the stock room and put into the diet room. Similarly, animals selected for transportation are taken from the stock room and then placed in the tumor room, where they remain until they are forwarded to collaborative investigators, or used for further maintenance of transplantable tumor lines.

Three induction experiments were started, in which diets containing specific carcinogens are fed for prolonged periods of time. For the Aflatoxin B_1 experiment, 21 male 5 week-old Buffalo rats weighing between 55 and 80 grams were selected. They were fed the experimental and control diets alternately: 4 weeks Aflatoxin B_1 containing diet followed by 1 week stock diet, for 4 periods or a total of 16 weeks on the experimental diet. Body weights and food consumption were measured weekly, as were body weights and food consumption of control animals. Present results indicate that the experimental animals increased in body weight at a somewhat lower rate than the control animals, and that this reduction in body weight rate roughly paralleled reduced food intake. Multiple hepatomas were induced. Assays of several enzymes was made on the primary tumors by Weinhouse's group.

A diet containing N-2-fluorenyldiacetamide at a low level is being fed to Buffalo-strain rats continuously. Both average daily food intakes, and average body weights for this group of animals (and their controls) are being determined. Several new hepatomas have resulted but we do not yet know how many are diploid.

Another experiment is currently underway to induce transplantable kidney tumors. Nitrosodimethylamine was injected intraperitoneally into 36 male Buffalo rats fed the standard pelleted feed. Half of the group was given a second injection. These animals are to be autopsied 9-12 months after the first injection or when kidney tumors have developed. No new kidney tumors have been obtained to date.

In order to maintain existing tumor lines, and assure ready availability of tumor-bearing animals to investigators collaborating on this project, all tumor lines have been transplanted at appropriate intervals. Approximate growth rates of the spectrum of hepatomas has been determined by measurements of tumor size as well as by the time between transfers.

Serial No. NCI-324
1. Laboratory of Biochemistry
2. Nutrition and Carcinogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Man Years:

Total:	2
Professional:	2
Other:	0

Project Description:

Methods: (a) Cooperative units who do not have the facilities for developing and producing their own hepatomas and other rodent tumors actively collaborate on problems of mutual interest. Tumor lines are selected by project officer that are most suitable to the needs of any particular research program.

(b) The chemical carcinogens used to induce rodent tumors are non-specific since a variety of neoplasms are induced by any given chemical. Not all neoplasms tested grow as transplantable neoplasms.

(c) The histology, biology, and biochemistry of primary and transplantable neoplasms are under continual study. The cooperating units use the most sophisticated methods and techniques applicable to the program under study where the main effort is to define and characterize the alterations between normal and neoplastic tissues. The liver and kidney provide abundant normal tissue to compare with primary and transplantable tumors from these respective tissues. The liver and/or kidney of tumor-bearing animals provide another tissue with which the influence of tumors on host tissue can be studied and compared with normal non-tumor-bearing liver tissue.

Major Findings: The development of well differentiated malignant hepatocellular transplantable carcinomas of different growth rate afforded an opportunity to study many areas of neoplasia. Most of these hepatomas metastasize to the lungs of tumor bearing hosts.

Efforts to induce transplantable hepatomas more nearly like liver were continued. Eight compounds, non-liver specific carcinogens, have been studied in rats and found to induce primary hepatomas that have been transplanted (4). The chemicals were the following: N-2-fluorenyldicetamide, N-2-fluorenylphthalamic acid, 2-(4'-methyl)-benzoylaminofluorene, N,N'-2,7-fluorenylenebis-2, 2,2-trifluoroacetamide, N,N'2,7-fluorenylenebissuccinamic acid, 2-ethylnitrosaminofluorene, 4'-fluoro-4-acetylaminobiphenyl, and 2,4,6-trimethylaniline. This is not an exclusive list. Several histologic types of hepatomas have been developed. These hepatomas vary many-fold in rate of growth. Some, although not all, poorly or undifferentiated tumors, grow more rapidly than well differentiated ones.

As described last year, studies of DNA concentration and incorporation of ^{14}C-labeled thymidine were compared in normal and regenerating liver and in hepatomas of different growth rate by Lea, Morris and Weber. The DNA levels of 2-3 mg/gm of tissue found in control, host, and regenerating liver were not significantly increased in slowly growing hepatomas 7800 and 5123D. In a tumor of intermediate growth rate 7288C the mean DNA concentration was 177 percent of control normal livers; in two rapidly growing hepatomas 3683 and 3924A the increase was 250 and 211 percent. Thymidine incorporation into DNA closely paralleled growth rate of these hepatomas and thus becomes another way of measuring growth. An extension of this study to transplantable rat tumors was made with similar results.

Some of the pertinent information is as follows: Three transplantable kidney tumors have been developed. These adenocarcinomas of the kidney cortex were induced by feeding a diet containing 4'fluoro'4'biphenylacetamide. This chemical induces tumors at many other sites and the kidney tumors develop later than hepatomas and mammary gland tumors. It was found that by starting female rats 4 months of age on the carcinogenic regimen fewer mammary tumors developed and we were able to get kidney tumors. Each of the three kidney tumors has a different growth rate for which the fastest growing line requires about 3 months to grow to a useful size. The slowest growing line on the other hand takes 12 months to grow to a similar size. Studies by Katanuma and co-workers on glutaminase isozymes is under way.

As reported the metabolic studies of the fastest and slowest growing kidney tumor lines reveals (Lea, Morris and Weber) compared to control kidney cortex increased activity of the glycolytic enzymes hexokinase, phosphofructokinase, and pyruvate kinase. The activity of the gluconeogenic enzymes-glucose 6-phosphate and fructo-1,6-diphosphatase were decreased. Lactate production from glucose was very low or undetectable in fortified homogenates of rat kidney cortex but in the fastest growing kidney tumor there was a marked lactate production from glucose. The glucose-6-phosphate and 6-phosphogluconate dehydrogenase were increased. Thymidine-2-^{14}C incorporation into DNA was greater for the fastest growing kidney tumor than for the slowest growing kidney tumor. The increases in the ratios of glycolytic to gluconeogenic enzymes in kidney tumors and the rise in thymidine incorporation, resemble the alteration previously observed in hepatomas and thus further emphasizes similarities of neoplastic tissue in another tumor model system.

The enzyme glutaminase involved in detoxication of ammonia to urea and its regulation in the kidney may be important in kidney neoplasms. Therefore, collaborative studies of kidney tumors were undertaken with Dr. Katunuma. So far, these studies have disclosed that the rapidly growing rat kidney tumor contains high activities of the phosphate independent and also the phosphate dependent glutaminase isozymes. The specific activity of the phosphate dependent isozyme was 5 times higher in the tumor than it was in the normal kidney. Phosphate independent glutaminase isozyme of liver and brain is activated by N-acetyl glutaminate but the normal kidney one is not. The latter however, is strongly activated by a peptide fraction isolated from kidney, but the liver and brain phosphate in dependent glutaminase isozyme is not

activated by the kidney peptide fraction. The kidney tumor phosphate independent glutaminase isozyme is activated by both N-acetyl glutaminate and the kidney peptide fraction. It thus seems that in this kidney tumor line the enzyme is not a mixture of the two isozymes but has the binding sites for both activators. A comparison of the slowly growing kidney neoplasm with the activity of these glutaminate isozymes in the rapidly growing kidney tumor and their possible role in the regulatory mechanisms of glutaminate metabolism is in progress. Further studies of these enzymes to hepatomas of different growth rates. Definitive results are not yet available.

Collaborative studies of chromosomes in a spectrum of 41 hepatomas with Dr. Nowell reveals that only 2 hepatomas have the normal diploid number of chromosomes and normal karyotype although five other hepatoma lines had a diploid number of chromosomes but some minor alterations in their karotypes. One fast growing tumor was hypodiploid. Several slowly growing tumors were hyperdiploid and or aneuploid.

Defective control of lipogenesis and/or fatty acid synthesis has been found by Elwood and Morris; and Sabine, Abraham, and Morris independently in five different transplantable hepatomas. Feeding fat deficient diets to control rats markedly increased fatty acid synthesis from acetate-1-^{14}C but did not influence fatty acid biosynthesis in the hepatomas. The dietary regimens that greatly altered metabolic and enzymic activities in host or control livers were found not to affect enzymic activities of hepatomas. This finding may indicate a functional aberration that is fundamental to the neoplastic process.

In collaboration with Dr. Potter, Pitot and Watanebe seven diploid, one aneuploid and one tetraploid transplantable hepatoma lines have been assayed for several key liver enzymes at 12, 30 and 60% dietary protein with standardized light and dark periods with food during dark periods. Marked oscillatory patterns were noted for α-ketoglutarate transaminase in both host-liver and hepatomas, but serine dehydratase, glucose-6-phosphate dehydrogenase and citrate cleanage enzyme did not show cyclic changes in either host liver or hepatomas.

Compared to the responses of enzyme activity to increased dietary protein in host liver or normal liver, the hepatoma enzyme activity tends to be either "non-induced", "derepressed", or less sensitive to environmental factors than normal liver.

One hepatoma 9618A has glycogen within the normal range. The others studied contain very low levels of glycogen. There appeared to be a reciprocal relationship between the activities of serine dehydratase and glucose-6-phosphate dehydrogenase. Both these enzyme activities from the various tumors fall well within the area indicated by the values for normal rat liver under the various environmental conditions but the hepatomas appear to resist alterations from their location within the range of values of normal liver. Much variation in these enzyme activities was noted among the various diploid transplantable hepatomas.

Significance: Hepatomas of different growth rates provide for the first time a spectrum of biological models which are furnishing the tools to examine biochemical differences related to different degrees of malignancy. The value and usefulness of these tumors for such studies is recognized by many of the foremost cancer investigators in this country and abroad. Hepatomas having many biochemical and biological characteristics similar to normal liver are slowly growing and have less capacity to incorporate thymidine into DNA.

Degradative enzymes and kinases in cell-free systems were not rate limiting. The very slowly growing hepatomas, Webb and Morris, have more organized endoplasmic reticulum and fewer free ribosomes. Some of the free polyribosomes will bind to membranes from normal liver while free polyribosomes in hepatoma 7800 do not bind to membranes. It is clear that graded biochemical alterations occur in the spectrum of hepatomas. Some biochemical parameters increase with growth rate, others decrease while still others are neither increased nor decreased. Biochemical characterizations, however are much more specific than morphological characterization. No other spectrum of tumors has ever been developed which offes so many possibilities to study the mechanism(s) of carcinogenesis and variations in malignancy. Many-fold differences in growth rate occur yet the development of identical experimental hepatomas has not been noted. Many avenues of attach of the problem of carcinogenesis are indicated and are being actively pursued. Studies of this spectrum of tumors has had a great impact on our thinking regarding experimental cancer. The development of transplantable rat kidney adenocarcinomas with differing growth rates has added a second biological model system which so far shows many similarities to the hepatoma models and is expected to prove useful in studying the alterations involved in the development of neoplasia.

Proposed course of project: New and continued collaborative studies have been set up to explore more control mechanisms as a means of obtaining a more comprehensive understanding of experimental cancer in both liver and kidney neoplasms. These have been most productive and promise to continue to be productive for the forseeable future. Many new collaborative studies were activated during the year.

Honors and Awards: None

Publications:

1. Morris, H. P.: Iodine-physiological aspects. In Clark, G. L. and Hawley, G. G. (Eds.): Encyclopedia of Chemistry. New York, Reinhold, 1966, pp. 565-566.

2. Wheeler, G. P., Alexander, J. A., Hill, D. D., and Morris, H. P.: Effects of partial hepatectomy upon the metabolism of formate-^{14}C in vivo by host liver and Morris hepatoma 5123-C. J. Nat. Cancer Inst. 36: 709-715, April 1966.

3. Morris, H. P. and Wagner, B. P.: Chemical induction of transplantable hepatic rat tumors having different rates of growth. In Busch, H. (Ed.), Vol. 4, Chapter 4: Methods in Cancer Research. New York, Academic Press, 1968, pp. 125-152.

4. Miyaji, H., Morris, H. P. and Wagner, B. P.: Histological study on some primary and transplantable hepatic tumors in rats. In Busch, H. (Ed.), Vol. 4, Chapter 5: Methods in Cancer Research. New York, Academic Press, 1968, pp. 153-176.

5. Kwan, Sau-wah, Webb, T. E. and Morris, H. P.: Diversity and nature of ribosomal pools in hepatoma 7800 and host liver. Biochemical J., 1968. In press.

6. Rogers, L. A., Morris, H. P. and Fouts, J. R.: The effect of phenobarbital on drug metabolic enzyme activity, ultrastructure, and growth of a "minimal deviation" hepatoma (Morris 7800). J. Exp. Pharmacol. and Exp. Therap. 157: 227-244, 1967.

7. Marks, F., Süss, R., Hecker, E., and Morris, H. P.: Metabolism of estrone by microsomes from liver and transplantable hepatocellular carcinomas (including "minimal deviation" hepatomas 5123C, 7800 and 9121). Z. Krebsforsch. 69: 361-369, 1967.

8. Ohe, K., Morris, H. P. and Weinhouse, S.: β-hydroxybutyrate dehydrogenase activity in liver and liver tumors. Cancer Res. 27: 1360-1371, 1967.

9. Elwood, J. C. and Morris, H. P.: The absence of adaptive hepatic lipogenesis in hepatoma 9121. J. Lipid Res., May 1968. In press.

10. Sabine, J. R. Abraham, S. and Morris, H. P.: Defective control of fatty acid synthesis in four transplantable rat hepatomas: Numbers 5123C, 7793, 7795, and 7800. Cancer Res. 28: 46-51, 1968.

11. Nowell, P. C., Morris, H. P., and Potter, V. R.: Chromosomes of "minimal deviation" hepatomas and some other transplantable rat tumors. Cancer Res. 27, 1565-1579, Sept. 1967.

12. Lea, M. A., Morris, H. P. and Weber, G.: Precursor incorporation into DNA in liver and kidney tumors of different growth rate. Cancer Res. 28: 71-74, 1968.

13. Lo, C., Cristofalo, V. J., Morris, H. P. and Weinhouse, S.: Studies on respiration and glycolysis in hepatic tumors of the rat. Cancer Res. 28: 1-10, 1968.

14. Drews, J., Brawerman, G. and Morris, H. P.: Nucleotide sequence homologies in nuclear and cytoplasmic ribonucleic acids from liver and hepatomas. Europ. J. Biochem. 3: 284-292, Jan. 1968.

15. Unuma, T., Morris, H. P. and Busch, H.: Comparative studies of the nucleoli of Morris hepatomas, embryonic liver, and aflatoxin B_1-treated liver of rats. Cancer Res. 27: 2121-2133, Nov. 1967.

16. Drews, J., Brawerman, G., and Morris, H. P.: Nuclear RNA patterns in rat liver and hepatomas. Naturwissenshaften 54: 619-620, 1967

17. Busch, B., Arendell, J. P., Morris, H. P., Neogy, R. K. and Schwartz, S. M.: Base composition of RNA of nuclear fractions obtained by sequential extraction with saline solutions. Cancer Res. 28: 280-285, Feb. 1968.

Serial No. NCI-333
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biochemical studies on normal and leukemic leukocytes.

Previous Serial Number: Same

Principal Investigators: W. H. Evans, S. R. Himmelhoch, M. G. Mage, and E. A. Peterson

Other Investigators: None

Cooperating Units: Cytochemistry Section, Laboratory of Biochemistry NCI

Man Years:

Total:	1
Professional:	3/4
Other:	1/4

Project Description:

Objectives: To investigate the biochemical factors regulating growth, maturation, and the phagocytic capacity of leukocytes especially with regard to the arrested maturation and decreased phagocytic activity of leukocytes from myelogenous leukemia; to exploit any biochemical differences observed between normal and leukemic leukocytes for purposes of improving diagnosis and treatment of leukemia. The development of myeloperoxidase in the course of leukocyte maturation is being studied as an exemplar because of its favorable biological and physical properties.

Methods: Leukocytes are isolated from bone marrow, blood and inflammatory exudates by 1xg sedimentation, glass bead columns, and immunologically-treated plastic foams (see NCI-396). Absolute and differential cell counts are determined. Oxygen uptake by intact cells is determined by manometric methods. Enzymes are assayed spectrophotometrically. Radioactive compounds are assayed using liquid scintillation counting techniques. Subcellular fractions are prepared by differential centrifugation, and further fractionated by chromatography and electrophoresis. Immunological reagents

are prepared from the purified fractions for following the development of related material during maturation of the cells.

Major Findings: Methods of extraction and fractionation have been devised which provide highly purified myeloperoxidase from the bone marrow cells of the guinea pig. Multiple components of this enzyme or additional enzymes of related specificity have been found. The purified protein will be used to produce an immunological reagent for studying the interrelationships of these components and the time course of assembly of the enzymes during differentiation.

Significance: The first steps have been initiated in following the course of white cell differentiation at a molecular level. Myeloperoxidase, the enzyme chosen as a model for these studies, increases in activity during cell maturation and is involved in one of the main functions of the mature granulocyte, namely, phagocytosis. In leukocytes obtained from acute myelogenous leukemic patients, myeloperoxidase activity is absent and the cell's capacity for phagocytosis is greatly diminished.

Proposed Course of Project: The time course and subcellular localization of assembly of the myeloperoxidase molecule during differentiation will be studied by identifying the order of appearance of its protein, heme, metal, and activity, using the techniques outlined.

Honors and Awards: None

Publications:

Evans, W. H., and Rechcigl, M., Jr.: Factors influencing myeloperoxidase and catalase activities in polymorphonuclear leukocytes. Biochim. Biophys. Acta 148: 243-250, 1967.

Serial No. NCI-375
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June30, 1968

Project Title: Homogeneity and Structure of Proteins.

Previous Serial Number: Same

Principal Investigator: A. S. Ralph Himmelhoch and Elbert A. Peterson
B. O. Wesley McBride

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1
Professional:	1
Other:	0

Project Description:

Objectives: To develop methods and procedures for the analysis and separation of proteins and to apply these methods to the purification of proteins and the study of their structure-function relationships.
A. The purification and structural study of leucine aminopeptidase.
B. The investigation of the possibility that disulfide bonds may play a role in the cross linking of vertebrate tropocollagen.

Methods: Fractionation techniques used include cellulose ion-exchange chromatography, column and disc electrophoresis, gel filtration, and salt precipitation. Fractions obtained are examined by electrophoretic and ultracentrifuge techniques, by atomic absorption spectroscopy, and by a variety of methods for analysis of enzyme activity.

Major Findings: A. The isolation of leucine aminopeptidase from swine kidney has been simplified and reduced to a routine procedure. The product has been examined extensively by techniques developed to detect endopeptidase contamination and has been found to be free of such activity. It is, thus, the only available preparation of this enzyme entirely suitable for use

in peptide and protein sequence analysis. Other laboratories at the NIH have used the enzyme extensively in sequence determination and confirm these results.

The relationship of metals to the activity of the enzyme has been reexamined. The enzyme contains, by analysis, from 2.6 to 4 gram atoms of zinc/mole of protein (300,000 g). It is inhibited by a wide array of chelating agents with an affinity for Group IIb metals. The zinc can be removed from the enzyme with concomittant loss of activity or replaced by cadmium to give an inactive form of the enzyme, which can be reactivated by replacement of cadmium by zinc. The role of Mn activation of the enzyme has been found to result from the replacement of a portion of the native metal (zinc) by Mn^{2+} under the conditions of the assay.

B. A report in the literature that a large fraction of rat skin collagen that remains insoluble during extraction with both NaCl and Na citrate is dissolved at neutral pH in the presence of mercaptoethylamine has been confirmed, and possible mechanisms of action have been investigated. Another thiol reagent, dithiothreitol, was also found to be effective, whereas the chelating agents, EDTA and o-phenanthroline, as well as a general reducing agent, sodium borohydride, were ineffective at pH 7.4.

Rat skin collagen oxidized by performic acid contains about 0.5 residue of cysteic acid per 1000 residues of total amino acids. The apparent cysteic acid content of this collagen was not altered when the denatured protein in 5M guanidine hydrochloride was passed through Sephadex G-200 to remove any low molecular weight contaminants. α_1 and α_2 chains isolated by CM cellulose chromatography contained only 0.2-0.3 residue of cysteic acid per 1000 residues of amino acid, whereas the dimers, β_{11} and β_{12}, have cysteic acid contents of about 1.0 and 0.3 residue per 1000 residues of amino acid, respectively.

The diagonal electrophoresis method of Brown and Hartley has been employed to separate the soluble peptides produced by the digestion of collagen by collagenase. A single, off-diagonal peptide was found after performic acid oxidation. Its high content of glycine, proline, and hydroxyproline indicate that it was probably derived from collagen, rather than from a contaminant, but it has not yet been demonstrated to contain cysteic acid.

Significance: A. Leucine aminopeptidase suitable for use in peptide and protein sequence determination has been made available. With the demonstration that it is a zinc metalloenzyme, rather than a magnesium- or manganese-requiring enzyme as formerly reported, all exopeptidases presently known and adequately studied are joined into a class of enzymes utilizing zinc as the native prosthetic group.

B. Further information concerning the structure of collagen, the most abundant mammalian protein, may shed some light on the nature of changes associated with aging. Since connective tissue forms a barrier between the circulation and tissues, as well as within tissues, such information may have relevance to factors involved in the growth and metastasis of neoplasms, also.

Proposed Course of Project:

A. The highly purified leucine aminopeptidase now available will be applied to the development of methods for rapid and automated peptide sequencing at the ultramicro level.

B. Peptides resulting from the digestion of collagen by several proteolytic enzymes will be further fractionated by diagonal electrophoresis, as well as by ion-exchange chromatography, to determine whether cystine is an integral part of collagen and functions in cross linking. Labeling with radioisotopes, in vivo, may be employed to aid identification and localization of cystine peptides.

Honors and Awards: None

Publications:

A. Himmelhoch, S. R. and Peterson, E. A.: Preparation of leucine aminopeptidase suitable for use in protein sequences determination. Biochemistry, in press, 1968.

B. None

Serial No. NCI-390
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Structure and function of an extracellular ribonuclease from Bacillus amyloliquefaciens.

Previous Serial Number: Same

Principal Investigators: Robert W. Hartley, Edward A. Barker and John R. Smeaton, Visiting Fellow

Other Investigators: None

Cooperating Units: J. C. Keresztesy, Section on Vitamin Metabolism Laboratory of Nutrition and Endocrinology, NIAMD.

Man Years:

Total:	3
Professional:	2-1/2
Other:	1/2

Project Description:

Objectives: To further purify, characterize, and investigate this enzyme and its natural inhibitor by physical, chemical, and biological techniques. To develop methods of culture, genetic manipulation and genetic analysis so that the organism and its product enzyme-inhibitor pair may be applied to a broad study of enzyme structure, function, and synthesis.

Methods Employed: B. amyloliquefaciens (strain H and derivatives and several other strains all of which were formerly considered to be strains of B. subtilis) are grown in synthetic media in a variety of batch and continuous flow fermentations. Isolation of the enzyme involves: 1) adsorption onto P-cellulose from acidified culture medium, followed by stepwise elution, 2) desalting by Sephadex chromatography, 3) gradient chromatography on CM-cellulose. The inhibitor is obtained from the cells after lysis or cold shock treatment. It is purified by ammonium sulfate and acid precipitations, DEAE-cellulose and Sephadex. G-75 chromatography and by specific adsorption to enzyme immobilized by covalent attachment to cellulose or agarose.

Work on the determination of the amino acid sequence of the enzyme (and the inhibitor, when sufficient material is available) involves amino acid analysis, enzymatic digestions, peptide separations, and peptide sequencing by the combined PTH-dansyl techniques. Quantitative amino acid analysis of peptides is being done by gas-liquid chromatography of trifluoroacetyl-butyl ester derivatives. PTH and "dansyl" amino acid derivatives are identified by thin layer chromatography on silica gel and polyamide layers. Chemical modification of the enzyme, such as succinylation, is used to reduce the number of peptides obtained on enzymatic digestion. Peptide sequencing by exopeptidase digestion is also being used. C-terminals are being determined by a technique that involves specific tritiation of the C-terminal amino acid. Peptide separations are primarily by 2-dimensional chromatography electrophoresis on paper.

Bacterial mutants are obtained by chemical mutagenesis of vegetative cells and by gamma-irradiation of spores. A variety of techniques are used to isolate ribonuclease-, nutritional-, and antibiotic-resistant mutants.

Major Findings: The enzyme has been found to undergo a reversible thermal transition that synchronously affects ultraviolet absorption, optical rotation, and gel filtration behavior. From this and other evidence, it has been concluded that the transition is highly cooperative and involves extensive disorganization of the native structure.

The transition temperature and the ΔH of the transition are affected by urea, by extremes of pH, and in different ways by cupric and sulfate ions. They are not affected by phosphate or substrate analogs. A fully succinylated derivative is inactive but maintains essentially the native conformation, with reduced stability.

At least three of the 6 or 7 tyrosines are buried in the folded form and inaccessible to solvent. At least one of the tryptophans is also buried.

The molecular weight of the enzyme has been redetermined as 12,100.

The inhibitor has been obtained in relatively pure form, as judged by disc electrophoresis and by experiments involving complexing with the enzyme. It has been established that the inhibition involves the formation of a one-to-one complex between enzyme and inhibitor. The reaction is reversible in urea solution and with appropriate manipulation both activities may be recovered from the complex.

Progress continues in the effort to determine the amino acid sequence of the enzyme. The development of micro methods for "dansyl", Edman, and gas-liquid chromatographic techniques has made practical the use of peptides isolated from paper maps. Several short sequences

are now known and the amino acid compositions of several tryptic peptides obtained. The N-terminal (alanine) was previously reported, and the C-terminal (arginine) has now been established.

Crystals of the enzyme may be obtained in either high or low salt concentration.

A B. subtilis phage (SP-10) has been adapted to grow in some of our ribonuclease-producing strains, but others appear to be resistant. Preliminary efforts to transduce nutritional factors have not been successful.

Significance: The small size, stability, absence of disulfide bridges, crystallizability and easily measured activity suggested this enzyme as an excellent choice for a long-range study of the relation between the structure of an enzyme and its function.

The nature and extent of the thermal transition give strong support to the concept that the three-dimensional structure of an enzyme may be completely determined by its amino acid sequence. The simplicity and all-or-none nature of the transition should make it useful for thermodynamic analysis of the factors involved in maintaining a folded protein structure.

The existence of a low molecular weight protein which specifically inhibits the enzyme by complexing with it offers a model of protein-protein interaction. The production of both by the same organism is also of biological interest.

Proposed Course of Project: Attempts to improve production and purity of enzyme and inhibitor will continue.

Further demonstration of the state of purity obtained, as well as further characterization, will be carried out with as many techniques as possible. Work on the thermal transition of the enzyme will be continued with particular reference to the accessibility of various residues in the two forms. It is hoped that a similar approach to the inhibitor and the complex will prove fruitful. The relation of particular substituents to stability, to activity, and to the interaction of the two proteins is of particular interest.

Determination of the primary structure of the enzyme is anticipated. That of the inhibitor is contemplated.

Growth of crystals large enough for x-ray diffraction studies will be attempted.

Comparison studies by the same techniques will also be applied to the homologous proteinsproduced by other, independently isolated, strains of B. amyloliquefaciens. It is a hope that similar studies can be applied to altered enzymes and cross reacting material produced by genetic variants of these strains and/or by introducing amino acid analogs.

Honors and Awards: None

Publications:

Hartley, R. W.: A reversible thermal transition of the extracellular ribonuclease of Bacillus amyloliquefaciens. Biochemistry in press, 1968.

Serial No. NCI-396
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Fractionation and characterization of blood cells and bone marrow cells.

Previous Serial Number: Same

Principal Investigators: Warren H. Evans, L. H. Brubaker (Medicine Branch) NCI, M. G. Mage and E. A. Peterson

Cooperating Units: Cytochemistry Section, Laboratory of Biochemistry, NCI Medicine Branch, NCI

Man Years:

Total:	1-1/2
Professional:	1
Other	1/2

Project Description:

Objectives: To develop methods for the fractionation of normal and leukemic blood and bone marrow leukocytes as a basis for the study of the biochemical changes that underlie the maturation of normal leukocytes and the factors that lead to the arrest of maturation in leukemia.

Methods employed: Leukocytes are isolated from bone marrow, blood, inflammatory exudates, and other sources. Absolute and differential cell counts are determined. Circulating red cells are used for special purposes. Separation principles being expored include differential sedimentation and adsorption by ion-exchange or immunological agents.

Major Findings: Work continues on the further development of methods for separating leukocytes according to their stage of maturation. Erythrocytes, lymphocytes, monocytes and granulocytes from normal human blood have been separated, using our previously developed 1 x g sedimentation method. Peak tubes of the various blood cells showed the following purities: erythrocytes 96%, lymphocytes 75%, monocytes 93%, granulocytes 98%. Sedimentation of blood cells from a patient with chronic myelogenous leukemia resulted in a complete separation of erythroid from myeloid cells. The more immature myeloid cells (i.e., myelocytes, promyelocytes) sedimented more rapidly than mature myeloid cells (i.e., segmented and band granulocytes) yielding purities of up to 88% for the former and 90% for the latter.

A guinea pig bone marrow cell preparation enriched in myeloblasts and

immature granulocytes has been obtained, using a modification of the glass bead column cell separation method. Bone marrow cells are passed through a horizontal rotating column filled with serum-coated glass beads at room temperature. Up to 90% of the mature granulocytes adhere to such columns whereas the more immature granulocytes and blast cells pass through unadsorbed and are collected in the effluent. Most of the red cell precursors (60%) and monocytes (100%) are also removed by this process, which requires about 60 minutes to complete.

Studies are in progress on the factors which regulate enzyme formation and activation during the maturation of bone marrow granulocytes. Myeloperoxidase has been chosen as a model enzyme for these studies (see Project Number NCI-333).

Significance: The separation of circulating leukocytes at different stages of maturity in human leukemia offers a means of comparing these cell types biochemically with corresponding cells separated by the same method from normal blood and bone marrow.

The effectiveness of the horizontal rotating column of glass beads in removing mature granulocytes from guinea pig bone marrow suspensions should make it possible to achieve better resolution than previously obtained when these fractions are subsequently subjected to the 1 x g sedimentation procedure described in previous reports for this project.

Proposed course of research: Improvements in the 1xg sedimentation procedure will be looked for. New methods for cell separation involving immunochemical interactions, ion-exchange chromatography, and electrophoresis will be explored, particularly for use in conjunction with the sedimentation procedure.

Attempts will be made to develop a tissue culture system for studying the differentiation and maturation of immature cells isolated from bone marrow by the 1 x g sedimentation method.

Honors and Awards: None

Publications:

1. Peterson, E. A., and Evans, W. H.: Separation of Bone Marrow Cells by Sedimentation at Unit Gravity. Nature 214: 824-825, May 1967.

Serial No. NCI-5202
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Isolation, fractionation, and characterization of soluble native nucleoproteins.

Previous Serial Number: Same

Principal Investigators: O. Wesley McBride and Herbert A. Sober

Other Investigators: None

Cooperating Units: None

Man Years:

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Objectives: Determination of the role of nucleic acid-bound proteins in the regulation of the transcription and translation of genetic information.

Methods Employed: Tissue culture methods are utilized in the propagation of established mammalian cell lines in suspension and monolayer cultures. Metaphase cells are harvested from monolayer cultures by spontaneous detachment from glass in a low calcium medium. Cells in suspension cultures are arrested at mitosis by the addition of Colchicine or Vinblastine to the medium. Chromosomes are isolated from mitotic cells by methods employing differential and density gradient centrifugation.

Major Findings: The technique of single and double block with excess thymidine has been used to obtain a limited, but useful, degree of synchronization of cell growth. Other methods utilizing reduced temperature or cold shock were found to be much less successful.

Several methods of chromosome isolation have been compared. "Metaphase plates" and nuclei isolated in hexylene glycol and 1-2.5 x 10^{-4}M $CaCl_2$ contain significant amounts of adherent cytoplasm. The cytoplasmic contamination can be prevented by decreasing the calcium concentration

further and increasing the shearing force but concurrently there occurs deterioration of the morphology of the chromosomes. The complete removal of cytoplasm from chromosomes isolated in non-ionic detergents is found only in the presence of very low concentrations of divalent cations and is associated with deterioration of the chromosome morphology. Two recent methods proposed by Maio and Schildkraut and by Huberman and Attardi have been the most useful though some cytoplasmic contamination of chromosomes is present in these procedures also.

Significance: Chromosomes represent a biologically significant fractionation of native nucleoproteins. The proposed study should provide general information concerning the interaction of proteins and nucleic acids as well as the role of these interactions in the expression of genes.

Proposed Course: Several procedures currently provide reasonably good yields of chromosomes associated with significant amounts of non-chromosomal material. Methods involving differences in size, charge, and surface characteristics will be used to fractionate and purify these native nucleoproteins.

Honors: None

Publications: None

Serial No. NCI-5203
1. Laboratory of Biochemistry
2. Protein Chemistry Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of immunochemical and physical methods for the purification and characterization of immunocytes.

Previous Serial Number: SAME

Principal Investigators: Michael Mage, Warren H. Evans, and Elbert A. Peterson

Other Investigators: None

Cooperating Units: Cytochemistry Section, Laboratory of Biochemistry, NCI

Man Years:

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: The goals of this project are to develop methods for the isolation of immune cells from the non-immune cells of lymphoid tissue and to subfractionate the various classes of immunocytes in order to study the development of the different immunological activities and antibody proteins of the various immune cell types.

Methods Employed: Immunocytes are separated from non-immune cells by free sedimentation in the 1xg sucrose gradient system previously developed by Drs. E. A. Peterson and W. H. Evans (NCI-396) and by binding of immunocytes to antigen-coated polymer forms (NCI-396). Individual immune cells are assayed for γM-antibody production by the appearance of localized hemolysis in a gel method developed by Jerne. In conjunction with this assay, antiglobulin reagents are used to measure cells producing γG-antibodies. In the model system for binding of antibody producers to antigen-coated foams, the foams are first coated with immune globulin from rabbits immunized with sheep erythrocytes. Sheep erythrocytes are then passed through the foam, allowing them to bind to the antibody on the foam. Finally,

spleen cell suspensions from mice immunized with sheep erythrocytes are passed through the foam, allowing those cells with antibody on their surface to bind specifically to the immobilized erythrocytes. Preparation and assay of immunological reagents are done by standard immunological methods such as hemagglutination and complement fixation. The recently introduced method of Axen and Porath for coupling proteins to CNBr-activated polysaccharides has been adapted and used to prepare specifically purified antiglobulin antibodies on immunoabsorbents of IgG conjugated to agarose beads.

Major Findings: On sedimentation at 1xg in a sucrose gradient, most of the IgM and IgG antibody plaque-forming cells (PFC's) sediment more rapidly than do most of the non-PFC's. Thus, antibody PFC's have been enriched up to 18-fold over the unfractionated suspension. The technique has been improved by introduction of a 16-inch diameter sedimentation chamber to shorten sedimentation time, and by methods for handling and plating the cells that yield higher recoveries of PFC's and higher plating efficiencies.

Passage of spleen cell suspensions through antigen (sheep erythrocyte)-coated polyurethane foams results in the binding of most of the PFC's, which can subsequently be released by mechanical agitation and assayed. When spleen cells from a mouse immunized with sheep erythrocytes (SRBC's) were passed through such an antigen-coated foam, only 15% of all nucleated cells were bound, but these included 60 to 90% of the PFC's. When the spleen cells were not washed prior to passage through the foam, 27% of all nucleated cells were bound, including 70 to 80% of PFC's. When spleen cells from a mouse immunized with SRBC's were passed through a foam coated with human erythrocytes, only 6% of total nucleated cells and 6% of PFC's were bound. Nucleated cells from unimmunized mice were bound to the extent of 8-10%. The difference in binding of anti-SRBC spleen cells to SRBC-coated foam (15% of total nucleated cells) compared to their binding to a human erythrocyte coated foam (6%) indicates that approximately 9% of the total nucleated cell load of 1.5×10^7 nucleated cells were specifically bound to the SRBC-coated foam, over and above background. Although from 60 to 90% of the PFC's were bound, these constituted less than 1% of the bound nucleated cells. The other 99% of the specifically bound nucleated cells may be producers of non-hemolytic anti-SRBC antibodies, or may be cells that are passively coated with anti-SRBC antibody, causing them to bind specifically to SRBC's.

The studies on cell binding to antibody-coated foams are being extended to glass bead columns as a matrix for separation. Here, the beads were first coated with rabbit IgG, then with specifically purified anti-rabbit IgG, which in turn bound rabbit antibody that was specific for sheep erythrocytes. The capacity and efficiency of binding of SRBC's to such columns

was higher than for antibody-coated foam columns. However, when nucleated cells were passed through such glass bead columns, high non-specific binding remained a problem.

Significance to Bio-medical Research: A basic knowledge of cell growth and differentiation, including the development of immune reactivities, is needed to help in understanding infectious, degenerative, and neoplastic disease. Such knowledge must be sought on the cellular and molecular level. The enrichment of plaque-forming cells achieved in this work is a significant step toward the isolation of antibody-producing cells for detailed study of antibody synthesis.

Proposed Course: We plan to develop further our methodologies for separation of immunocytes and to study the relative roles of different immune cell types in such phenomena as adaptive primary and secondary immunity, transfer of homograft immunity, and delayed hypersensitivity. The separation of cells on the basis of surface antigens may be applied to the study of immunoglobulin gene expression during differentiation of heterozygotes.

Honors and Awards: None

Publications:

Mage, M. G., Evans, W. H., and Peterson, E. A.: Enrichment of antibody plaque-forming cells of spleen by sedimentation at unit gravity. Proc. Soc. Exp. Biol. and Med. 127: 478, 1968.

PHS-NIH
Summary Report
July 1, 1967 through June 30, 1968
Laboratory of Biology
NCI

The accomplishments of the Laboratory of Biology as best shown by the list of publications (see attached) have been even greater this year than last. Since last year's report there have been 62 papers by the staff that have been published or accepted for publication. Members of the laboratory collaborate freely with people throughout NIH and even throughout the world but of these 62 papers, 53 have members of the laboratory as senior authors. This high productivity argues for the free approach to basic research that is maintained in the laboratory.

Personnel

There are presently 71 people in the laboratory, 26 of whom are at the doctorate level. This includes two guest workers, Drs. Smith and Wakefield, in the Carcinogenesis Section. On March 31 of this year Dr. H. B. Andervont of this laboratory retired at the age of 70 after 36 years with the Public Health Service. No one has contributed more to our basic information on experimental cancer. Two of his assistants have been located in other laboratories and the third has retired. Dr. Ram Parshad returned to Punjab University in India on April 4, 1968 after two and a half profitable years here in the Tissue Culture Section. Not only did he accomplish much while he was here but got a research program underway on cytogenetic changes of cells in vitro which he will be able to continue actively back in his home university. At the end of this June Dr. M. Ben-David of the General Biology Section will return to Hebrew University after two years in this laboratory where he has been able to accomplish very well in collaboration with the Endocrinology Branch the aims of his planned program on prolactin when he arrived. He is now in excellent position to continue his work back in Jerusalem on the role of prolactin in mammary tumorigenesis not only in mice but also in women. Dr. Nelson Burstein, a Staff Associate, will be leaving the Carcinogenesis Section after completing his two year term that has been a profitable one.

During the past year Dr. Duc-Nuigeen transferred to the Carcinogenesis Section from the Etiology Division of NCI. Dr. Caroline Hardy is reporting to the Tissue Culture Section as an NIH Fellow on July 1, 1968, and on the same date Dr. Kurt Stromberg is reporting to the General Biology Section as a Research Associate. Limitation of space for the Tissue Culture Section and the General Biology Section after the anticipated move to Building 37 during the year will be a limiting factor on personnel in these two sections.

Summary of Research Findings

Significant progress has been made in a number of areas in the laboratory. Significant basic work on tumor viruses continues, particularly with the polyoma virus, the murine sarcoma virus, and the mammary tumor virus. These include EM studies, purification studies, and assay studies. New information has been obtained on tumor antigens and the role of the thymus continues to be a productive area of investigation. In addition, hepatocarcinogenesis in the rat and mouse is receiving considerable emphasis, as is the neoplastic transformation of cells in vitro.

Somewhat more tangential but ultimately of very basic importance to the cancer problem is the work going on in biochemical genetics or genetic control of protein synthesis. This is not only biochemical genetics of the mouse but also somatic cell biochemical genetics in the analysis of the proteins produced by the plasma cell tumors.

Some of these areas are summarized briefly as follows, with no attempt being made to include all areas but only to chose those which best illustrate the work of the laboratory. Investigators' names are purposely omitted here. They are in the bibliography.

Methodology in Tissue Culture

The Tissue Culture Section continues to play a leading role in methodology in tissue culture, in the development of the fluid suspension culture, the chemically defined media, etc. For the first time freshly explanted tissue has been grown in chemically defined media in sufficient quantity and good enough quality to be used for study before, during, and after the neoplastic transformation. Incidence of tumor production with this tissue is low and the time required for the neoplastic transformation ranges up to 900 days. Perfectly normal highly differentiated tissues were seen after 1 year in culture as indicated by appearance of cells growing in the anterior chamber of the eye.

Neoplastic Transformation of Cells in vitro

The observations that neoplastic conversions of mouse embryo cells occur reproducibly after 90-108 days of culture in chemically defined media (NCTC 135) plus 10 percent horse serum have been confirmed. Fetal calf serum in place of horse serum prevents or at least delays this conversion. Yet calf serum acts like horse serum in bringing about early conversion. The longer cells have been kept in the fetal calf serum the longer it takes for conversion when they are transferred to horse serum. It appears that fetuin, the major protein component of fetal calf serum, is not the factor preventing the conversion. These observations hold for cells from several mouse strains and also from Albany rats, but it takes longer for the conversion to occur in the rat cells.

There was an increase in chromosomal abnormalities at about the same time as the neoplastic conversion. Cells showed greater chromosomal stability in fetal calf serum than in horse serum but there was also greater stability in calf and bovine serum than in horse serum. Chromosomes of cells from the germ-free ALB-2T mouse were more stable than those of the C3H mouse, but these

cells did undergo neoplastic conversion. Chromosomes of rat cells in vitro are more stable than those of the mouse. Yet there has been no chromosomal abnormality linked with the neoplastic change.

A new lead on biochemical differences that may be related to the neoplastic change is the observation of a qualitative as well as a quantitative difference in the RNA methylase complement of paired neoplastic and non-neoplastic cultured cell lines. A glycoprotein present in wheat germ has been reported elsewhere to be a specific agglutinin for malignant cells, but work in this laboratory failed to show any correlation between malignancy and agglutination activity.

In vitro transformation of mouse cells with SV40 virus has been accomplished. These transformed cells contain tumor specific T antigens that can be demonstrated by immunofluorescence. The transformed cells grow as tumors in mice of the strain of origin, whereas cells from lines not transformed by the virus do not.

Viruses in Tissue Culture Cell Lines

Recently a C-type virus was isolated from a C3H minced mouse embryo tissue culture on completely chemically defined medium. The virus has since been found in other cultured lines, both neoplastic and non-neoplastic. By complement fixation and neutralization tests the virus was shown to belong antigenically to the Gross leukemia virus group. It can infect virus-free cultures from various mouse strains and it can infect mice, but it has not induced leukemia in mice of any of the three strains, BALB/c, C3H, or DD into which it was injected. It has not been shown to produce the neoplastic change in cultured cells. It will function as a "helper" virus in the recovery of Moloney sarcoma virus.

A particles were also found in some of the cultured cell lines but, unlike the C particles, the A particles were not found in any passage cultures.

Studies on Murine Sarcoma Virus (MSV)

Much of the work in this area has been directed toward the "rescue" of defective MSV in the mouse. This can be accomplished by a number of leukemogenic viruses of the mouse. The resulting lesion is an atypical granuloma. A quantitative assay for murine leukemic viruses by their ability to rescue MSV genome in MSB-1 cells has been worked out in vitro. This assay is 5 times more sensitive than complement-fixing tests and requires less than 21 days. It also does not depend upon other reagents such as complement and specific antisera which are often difficult to obtain.

Mammary Tumors in Mice

The mouse mammary tumor continues to serve as a model for the study of viral, genetic, and hormonal factors in the causation of cancer. That there are two forms of the virus, the MTV and NIV which, although they look alike, differ in their mode of transmission, is well established. The MTV, however, is not solely responsible for the mammary tumors and the NIV not

solely responsible for the hyperplastic nodules. Strain BALB/c in which neither can be demonstrated under the EM or with the Ouchterlony immunodiffusion test or the Nandi nodule inducing test has an incidence of approximately 40 percent mammary tumors in old breeding females and they also have hyperplastic nodules. In other strains with and without the MTV the number of hyperplastic nodules and age of occurrence varied with potency of MTV, presence of NIV, gentotype, and whether or not the females were bred.

Using the Ouchterlony immunodiffusion test for the mammary tumor virus, it has been shown that only about 50 percent of strain A females give a positive test, which is approximately the incidence of mammary tumors in the strain. These females will be kept to see if their later development of mammary tumors correlates with this test for the mammary tumor virus.

Work is being initiated in the laboratory and in collaboration outside the laboratory to apply the knowledge of the various biological factors in the causation of these murine tumors and the methods of concentrating virus and testing for it to the discovery of factors in the causation of human cancer.

Study of Prolactin and Its Relation to Mammary Tumors

There have been three aims in this study: 1) to determine the mechanism of action of perphenazine in production of prolactin in rats; 2) to study the influence of the mammary tumor virus on the mammotropic effect of perphenazine in mice; and 3) to demonstrate the existence of human prolactin. All three of these aims have been accomplished. It was shown that in the rat perphenazine stimulated endogenous secretion of prolactin acting within the pituitary; it was shown that the mammary tumor virus potentiates the mammotropic effect of perphenazine; and the existence of human prolactin as distinct from growth hormone was demonstrated. This opens the way to further studies on the relationship between prolactin and mammary cancer in mice and the investigation of a possible relationship between prolactin and breast cancer in women.

Hepatocarcinogenesis

Carbon tetrachloride, a well known hepatocarcinogen for the mouse, is now being shown to be carcinogenic for the liver in rats. Older rats up to one year of age are more susceptible than younger rats in contrast with the higher response of younger rats to fluorenamine compounds.

Histogenesis of cholangiocarcinomas in hamsters fed N-2-fluorenylacetamide and N-2-fluorenyldiacetamide has been followed through cholangiofibrosis and cirrhosis to the eventual development of well differentiated cholangiocarcinomas. The same response has been noted in Fisher rats fed 3'-methyldimethylaminoazobenzene. Carcinomas of the parenchymal cells induced in rats with N-2-fluorenyldiacetamide are preceded by hyperplastic lesions. These hyperplastic lesions do not grow progressively on transplantation, but the later small, well-differentiated carcinomas do grow on transplantation.

Growth hormone increased the incidence of cirrhosis and carcinomas of the liver in castrated male and female rats fed N-2-fluorenyldiacetamide, but it had no effect on the response of intact rats to this carcinogen.

Role of the Thymus and Immunosuppression in Oncogenesis

In work with the polyoma model system it has been shown that lymphoid cells from the spleen of immunized syngeneic donors are effective in repressing primary neoplasms. Recently it has been found that sensitive syngeneic spleen cells are effective if sonicated, which opens the possibility of pinpointing the reactive antigenic site of the target cell. C3H/Bi mice that had been made tolerant to C3H/Lw tissue by i.v. inoculations of spleen cells, subsequently accepted sensitive C3H/Lw cells given 30 days after polyoma virus infection, and this reduced the number of polyoma induced tumors.

Where transplantation antigens and the immunocompetence of the host are major determinants in the induction of neoplasms, a reduced frequency or lower latency period would be expected from thymectomy. In studies on lung tumors induced with methylcholanthrene and with urethan, and also on hepatomas, no effect of thymectomy could be demonstrated. The latter is of interest since there is some indication that carcinogen induced hepatomas in both the rat and mouse contain transplantation antigens. MTV induced mammary carcinoma in C3H mice are known to contain transplantation antigens and in these a slightly negative effect of neonatal thymectomy was observed when the MTV was given at 21 days so that tolerance to the virus could not develop.

Biochemical Genetics

Biochemical and immunochemical methods are being developed with special emphasis on the mouse urinary proteins and catalase. A new finding regarding the urinary protein complex is that it is made up of 4 components. These will be further characterized. At different pH's the protein components have different electrophoretic mobilities. Erythrocyte catalase has been studied. Genetic origin and sex were found to be the two main factors governing the level of activity of this enzyme.

Techniques learned here are being applied to a collaborative study of the ultrastructural localization of viral antigens, using horseradish peroxidase as an electron opaque marker.

The Plasma Cell Tumors

The study of the plasma cell tumors continues as a very strong area of investigation of genetic control of protein synthesis. There are about 150 different plasma cell tumors being carried in the laboratory and these synthesize and secrete specific forms of immunoglobulins, providing an experimental source of homogeneous immunoglobulin as well as the highly specialized and differentiated cells that synthesize the proteins. These genetic studies have revealed one locus known as the heavy chain linkage group that consists of 4 genes controlling the heavy chains Ig, -A, -F, -G, and -H. All inbred strains carry only one of the heavy chain markers, F_1 hybrids can carry two,

but in studies of wild mice from a number of locations in this Country, Japan, and Taiwan, some have been identified with 3 markers. Apparently most of the inbred strains carry the same locus controlling light chains.

In screening a number of myeloma proteins for anti DNP activity one protein, MOPC315 was found that strongly precipitated DNP and TNP. This strong precipitating activity of 315 has been confirmed and an intensive study of this protein has been started. Subunits of the protein are being characterized, efforts at crystallization are begun, as is an effort to amino acid sequence the protein.

Plasma cell tumors are now being grown in vitro in this laboratory and it has been shown that they continue to secrete the same immunoglobulins in vitro that they did in vivo. Work is now underway on the cloning of these cells. Upon accomplishing this, studies of fusion of cells to observe the effect of mixing of genotype on the types of proteins produced will be begun. The unusual MOPC315 is now growing in vitro, thus opening the way for a number of interesting studies with this unusual protein.

Studies on tryptic digestion fingerprints of γM heavy chain mouse macroglobulins indicate that there is greater variation in the pattern of γ chain than in heavy chains of other immunoglobulin classes. Attempts are being made to locate this variation. Attempts are also being made to negative stain γM molecules from plasma cell tumors and compare the fixed structure of a wide variety of γM molecules.

In studies of γA myeloma proteins produced by these plasma cell tumors two different forms of γA myeloma proteins have been found. One resembles the γA immunoglobulins described in man, rabbit, and the horse, and can be isolated from mouse serum and ascites. The other γA protein has not been described in other species. It is found in the serum and is excreted in the urine of mice bearing certain plasma cell tumors.

Transfer RNA has been isolated and studied from mouse plasma cell tumors, primarily those producing only kappa-type light chains. Different tumors have different degrees of transcription of the DNA specific for each t RNA leu component. It appears that the genes are operating independently of one another rather than simultaneously, and whatever controls the DNA transcribing activity of tRNA, it operates at a level finer than one turning on or off all tRNA leu.

Amino Acid Sequence Studies

A collection of about 100 kappa urinary proteins produced by plasma cell tumors are available for chemical characterization. Much progress has been made in analyzing the structure of the kappa urinary proteins through amino acid sequence analysis. Attention has been focused on the amino-terminal peptide. A lambda type of mouse myeloma light chain has been described for BALB/c mice and much progress has also been made in the amino acid sequence of these proteins, particularly the carboxyl-terminal portion and the amino-terminal portion. They were found to be identical. Rabbit

light chains have also been analyzed. Comparison of the carboxyl terminal sequences of the rabbit, mouse, and human light chains shows a striking homology.

Induction of Tumors in Germ-Free Mice

Plasma cell tumors are not induced as readily in germ-free mice as in conventional mice. This would indicate that other environmental factors play a role in plasma cell carcinogenesis. It has been shown that subcellular centrifugates from plasma cell leukemias and reticulum cell sarcomas will increase the occurrence of plasma cell and reticulum cell neoplasms in BALB/c mice but not in DBA/2 mice.

Germ-free BALB/c mice do not develop lung tumors when injected with urethan as readily as do conventional mice.

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Serial No. NCI - 3200
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Factors influencing oncogenesis and the induction of new cellular antigens by the DNA and RNA viruses

Previous Serial Number: Same

Principal Investigator: Dr. L. W. Law

Other Investigators: Dr. R. C. Ting (NCI:LBgy-3218)
Dr. M. Stanton (NCI:LPath-516c)
Dr. Duc-Nuigeen (NCI:LBgy-3241)
Dr. Samuel Strober (NCI:LBgy-3243)
Dr. N. Burstein (NCI:LBgy-3230)
Dr. A. White (Albert Einstein College of Medicine)

Cooperating Units: NCI:LPath-516c

Man years: Professional - 1 (Dr. L. W. Law)
Others - 4
Total - 5

Project Description:

1. Rôle of thymus and immunosuppression in oncogenesis

a). Work has continued on the polyoma virus model system of attempts to repress tumor induction or already established neoplasms in the autochthonous C57BL host. Lymphoid cells from the spleen (as few as 30 x 10^3) are effective in repressing primary neoplasms providing these cells are obtained from an immunized syngeneic donor. These passive transfer experiments indicate that few lymphoid cells are needed. Assuming 70% of the splenic population are lymphoid in origin and that 10% of those sensitized as few as 2000 cells are therefore capable of a striking repression of neoplasms. The facts of ineffectiveness

of allogeneic or killed cells (by X-ray) and of serum fit with the concept of adoptive immunity.

b). Recently it has been found that sensitized, syngeneic cells of splenic origin are effective if sonicated. If this finding is repeated, it will indeed allow for the possibility of pinpointing the reactive antigenic site of the target cell. This work is being continued.

c). Since only sensitized syngeneic lymphoid cells were found effective in adoptive transfer, an attempt was made to use sensitized allogeneic cells by first rendering the recipient, polyoma virus-infected animal tolerant to the subsequent inoculation of cells. C3H/Lw mice are highly resistant to polyoma virus oncogenesis yet C3H/Bi mice are highly susceptible. These strains differ only in minor histocompatibility genes yet skin grafting and tumor transplantation between them do not succeed.

It was found that C3H/Bi mice made tolerant to C3H/Lw tissues by i.v. inoculations of 10×10^6 spleen cells at birth (tested by skin grafting) subsequently accepted sensitized C3H/Lw cells given 30 days after polyoma virus infection. These mice, in contrast to those given lymphoid cells at birth only or at 30 days only got strikingly fewer polyoma type tumors (See Table).

Table: Frequency of Parotid Gland Neoplasms in C3H/Bi Mice
Untreated, given sensitized C3H/Lw cells at 30 days (after polyoma virus injection), given sensitized allogeneic cells at 30 days subsequent to induction of tolerance at birth and given C3H/Lw cells at birth only.

Group	No. of Mice	% of Tumors
Polyoma virus only	12	80
PV + C3H/Lw cells AB	4	75
PV + " " at 30 days	25	70
PV + " " AB at 30 days	27	8

d). We have continued our investigations of the use of immunosuppressants, including thymectomy, in several systems - those neoplasms in which Transplantation Antigens (TA) are known to exist and in some commonly occurring tumors of mice - in order to determine if TA and the immunocompetence of the host are major determinants in the induction of these neoplasms. A reduced frequency of neoplasms and/or lower latency period should reflect this. The work with Dr. W. E. Heston (C:LBgy-3204) is now completed in which frequency at 5, 8, and 10 months autopsies and average number of lung tumors were determined in a susceptible mouse (C3H x A)F_1 and in a resistant hybrid, (C3H x B)F_1 following treatment with 0.25 mg methylcholanthrene at 2 months of age. No differences were found among the various groups, thymectomized or intact. Nor was there found any differences in frequency in several sublines of C3H mice in spontaneous hepatomas; autopsies here were performed at 9, 12 and 14 months. This latter finding is of interest since there is some indication that carcinogen-induced hepatomas in both the rat and mouse contain TA antigens.

Data are now complete also concerning the influence of thymectomy on the frequency of MTV induced breast adenocarcinomas in C3H/He mice. These neoplasms are known to contain TA antigen probably related to the intact virus particle. In the experimental "situation" in which tolerance to the virus or to new cellular antigens could not develop (that is MTV given at 21 days, rather than through the mother's milk), a negative influence of thymectomy was observed:

Thymectomized C3Hf/He	12/56 (21.4%)	13.7 months
Intact littermates	18/62 (29.1%)	13.2 months

Recently a group of Japanese investigators have found a negative influence of thymectomy on breast adenocarcinomas in strains of mice in which MTV did not play a rôle; underdeveloped mammary glands and alveolar nodules were observed in the thymectomized females. If true, this represents a function of the thymus unrelated to lymphoid tissues. We are pursuing this aspect.

2. Studies with MSV (Murine Sarcoma Virus)

Several phases of the work with the Moloney isolate of MSV are in progress and contemplated:

a). Pseudotype virus strains of defective MSV, from the MSB_1 epithelial neoplasm of Ting, have been rescued using several leukemogenic viruses such as MLV, RV, G (from AKR and C58) mice, X, from X-ray induced leukemia of C57BL/Ka mice, S from SJL reticular neoplasm. All of these strains induce the same histopathologic lesion in mice - an atypical granuloma with scatterings of neoplastic mesenchymal cells. They are tumors (massive tissue reactions) but do not grow progressively upon transplantation into intact adult syngeneic mice.

Under favorable conditions "rescue" of defective MSV has also been accomplished *in vivo* using the tissue culture grown MSB_1 cells inoculated directly into AKR recipients (containing G leukemogenic virus). Studies are continuing on the biologic, immunologic and serologic characteristics of the *in vivo* rescued pseudotypes. (With Dr. R. C. Ting:C:LBgy-3218).

b). From 30 or more MSV-induced tumors, 3 separate transplant lines of simple neoplasms have been selected through initial passages in X-irradiated and/or thymectomized mice. All are hemangiosarcomas and now grow progressively in intact, adult, syngeneic mice. One of these, XM 1B, does not produce infective new MSV - nor does it replicate leukemogenic virus. XM 1B is shown to have a virus-specified transplantation antigen which is of interest since this is a case of an RNA virus fraction apparently being incorporated within the neoplastic cell and specifying a new cellular antigen. Work is in progresss to define other characteristics of this antigen and possibly other antigens.

c). Attempts are being made also (with Dr. R. C. Ting, LBgy, and Dr. Duc-Nuigeen, C:LBgy-3241) to establish XM 1B in tissue culture to determine the presence of "defective" MSV. Two other transplantable lines of hemangiosarcomas produce minimal amounts of MSV as detected only in tissue culture and the polycation DEAE-dextran. Attempts will be made to free these simple neoplasms of MSV.

d). In contrast to the behavior of the simple XM 1B, hemangiosarcoma, the MSV-induced "granulomas" continue to replicate MSV; these are transplantable and grow progressively only in X-irradiated or thymectomized recipients. One of these XM-131 in the 15th to 20th transplant generations was studied for the presence of transplant antigens (TA) and was found to be strikingly inhibited in pretreated syngeneic host-pretreated with MSV, MLV or any syngeneic or allogeneic tumor tissues replicating MSV or MLV. Since progressive growth or inhibition in X-irradiated recipients was associated with anti-MSV antibodies, the effects of passive transfer of anti-MSV serum on the growth of this tumor was studied. It was found that 0.2 ml of antiserum given at the time of transplant and continued for 12 inoculations caused a strikingly reduced number of takes and inhibition of growth. Thus, this represents a case of TA antigen evoking a humoral response of antibody that is an effective inhibition of growth. In this situation it is impossible however to determine whether the TA is a new cellular antigen specified by MSV or is the action of the intact structural virus on the cell surface.

3. Restorative Effects of Thymosin

In collaboration with Dr. Abraham White (Albert Einstein College of Medicine) the restorative effects of the preparation Thymosin (See Goldstein, A. L., Slater, F. D. and White, A. Proc. Natl. Acad. Sci., U.S. 56: 1010, 1966) in neonatally thymectomized C57BL/Ka mice has been studied. The ability of 5×10^6 dissociated spleen cells from a) intact, b) thymectomized and c) thymectomized treated with Thymosin mice to produce graft-versus-host reactions (GVH) in newborn BALB/c mice was compared. These strains differ in their H-2 antigens - BALB/c being $H\text{-}2^d$ and C57BL, $H\text{-}2^b$. As seen in the table, Thymosin given 3 X/week beginning at 1 week of age - for 12 injections restored the ability of C57Bl-thymectomized mice to produce GVH. On the other hand there was not restoration of body weight, prevention of mortality nor restoration of the lymphocyte population. In this respect this preparation behaves much like the aqueous extracts obtained from neonatal syngeneic thymuses. See 1967 Annual Report.

Table:

Spleen Cells from C57BL	No. BALB/c Recipients	No. with GVH	Average Time to Death (days)
a) Control, intact	18	16 (88%)	15.8
b) Thymectomized	66	0	-
c) Thymectomized + 1 mg Thymosin x 12	40	25 (63%)	18.3

Attempts at restoration with Thymosin using other assay systems: skin grafting, tumor transplantation, Simonsen assay, etc. will be continued using also concomittant seeding with bone marrow and embryonic liver stem cells.

4. Studies with Anti Thymocyte Serum

Striking effects of ATS in BALB/c mice given MLV leukemogenic virus at 21 days was reported last year; the use of this immunosuppressant allows a study of the influence of immunologic depression on the induction of reticular neoplasms whereas thymectomy removes the organ intimately connected with the transformation. We have now found using 2-3 month old BALB/c mice given ATS 1 week prior to and 1 week subsequent to injection of MSV (MLV) that both intramuscular growths and lymphocytic leukemia appear in these treated mice whereas ATS alone or MSV (MLV) alone is ineffective; MSV (MLV) is the pseudotype MSV virus "rescued" by MLV from MSB_1 rat cells containing "defective" virus. A certain concentration of MLV would be expected in these preparations. Work is in progress to attempt to pinpoint the mechanisms involved - probably virus growth patterns and concentration of anti-MSV (MLV) antibodies.

Serial No. NCI - 3200
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

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Publications (con't).

Yosida, T., and Law, L. W.: Chromosomal alteration and the development of tumors XVII. Chromosomes of the leukemias induced by Moloney leukemogenic virus (MLV) infection. Japan Jour. Genetics, 1968. Accepted for publication.

Serial No. NCI-3202
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Genetic and structural study of myeloma immunoglobulins in mice and the pathogenesis of plasma cell neoplasia in mice

Previous Serial Number: SAME

Principal Investigator: Michael Potter, M.D.

Other Investigators:
Miss Rose Lieberman (I:LCI-15c)
Dr. E. Bruce Merchant (I:LI-151)
Dr. H. Metzger (A:A&R-8c)
Dr. J. Frederic Mushinski (C:LBgy-3222)
Dr. Phillip Periman (C:LBgy-3244)
Dr. Paul Anderson (C:LBgy-3240)
Dr. Leroy Hood (C:I-5010)

Cooperating Units:
Dr. Herman Eisen, Washington University School of Medicine
Dr. Myron Leon, St. Lukes Hospital

Man Years:
Total - 5
Professional - 1
Others - 4

Project Description:

Plasma cell tumors can be induced in high frequency in the inbred BALB/c strain of mice by mineral oils and solid plastics. Large numbers of plasma cell tumors can be isolated and maintained in transplant. Currently we carry about 150 different plasma cell tumors in the laboratory. These tumors synthesize and secrete specific forms of immunoglobulin molecules, thus providing an experimental source of homogeneous molecular forms of immunoglobulin as well as the highly specialized and differentiated cells that synthesize these proteins. The ultimate experimental aims are to learn: how the cells become specialized that is the mechanism of immunoglobulin gene differentiation and the relationship of this gene differentiation process to the neoplastic transformation.

Can we obtain a special vantage point about carcinogenesis by studying its development in a highly specialized cell system, where a number of specific changes occur, such as the differentiation of immunoglobulin formation? The approach to a complex problem such as this is to understand the biochemical basis of the specialization process. Ideally this could be directly examined if we could isolate the precursor cells and cells in various stages of progression to neoplasia. Experimentally this is not feasible as yet, and the main experimental approach is to examine the fully developed specialized neoplastic cell and its products. Two experimental approaches relevant to this aim are available with plasma cell tumors, the study of proteins synthesis and the

special protein product. This report will deal with experiments in two sections, those relating to protein synthesis and those relating to the study of the proteins. The various experiments will be discussed under the relevant study.

I. Studies on the immunoglobulins

The homogeneous specific immunoglobulin produced by an individual plasma cell tumor reflects a remarkable differentiation of the immunoglobulin genome. Currently this gene differentiation process can be broken down into 3 categories: 1. allelic differentiation, 2. class differentiation and 3. specificity differentiation. "Allelic differentiation" is the all or none selection of a single, autosomal immunoglobulin allele per cell for active protein synthesis. "Class differentiation" is the selection of one of the 5 heavy chain class genes per cell (γM, γA, γF, γG or γH) for protein synthesis. "Specificity differentiation" is the selection of a specific molecular form of immunoglobulin genes within a class per cell. Specificity differentiation establishes the unique structural characteristics that endow a protein to combine with specific ligands (haptens, conformational antigenic determinants, etc.). Specificity differentiation involves the development of a special protein synthesizing mechanism that establishes one unique light and one unique heavy chain per cell.

A. Allelic and class differentiation

Allelic differentiation: (includes studies on genetics of heavy chain linkage group and wild mice)

Only one genetic locus that controls immunoglobulin genes has been thus far identified. This locus known as the heavy chain linkage group contains at least 4 genes controlling heavy chains Ig-A, -F, -G and -H. Classic genetic approaches to the structure of the heavy chain linkage group have been pursued, including: 1. identification of individual genes, 2. attempts to order genes within the locus, 3. demonstration of linkage to other loci in the mouse and 4. demonstration of close linkage by crossover studies between genes within the locus.

The identification of individual genes has been by chemical and serologic characterization of the individual gene products. The accessible heavy chain markers are chemically, the tryptic peptide map pattern of the papain Fc fragment and serologically antigenic determinants found on the Fc fragments. A serologic system has been developed in collaboration with Miss Rose Lieberman (I:LCI-15c) and described in detail in the Advances in Immunology and the Cold Spring Harbor Symposia. Specific serologic markers (homologous allotypic antisera) are available for the products of the A, G and H genes of BALB/c. Since these publications we have attempted to obtain markers for the alleles of the C57BL/6 heavy chain linkage group. To do this a coisogenic strain

called BALB/c-2 has been developed by introgressively backcrossing the C57BL/6 heavy chain linkage group (identified by the "anti-2" homologous antiserum) into BALB/c. Currently this line is in the 15th backcross generation. Between the 6th and 8th backcross generations, backcross mice were mated with each other and 2/2 homozygotes were selected and used for plasma cell tumor induction. From roughly 30 tumors induced in these mice, Ig-A, -F and -H tumors have been identified. The myeloma proteins have been isolated from these and chemical and serologic characterization of the proteins has been carried out. Two identifying homologous antisera have been prepared, one specific for γH proteins and the other for γA proteins. Comparative tryptic peptide map studies of proteins derived from BALB/c and C57BL has shown peptide differences in each of the three comparisons. The largest number of peptide differences have occurred on the γH proteins the least in γF.

Tumors have also been induced in heterozygotes BALB/c-2 backcross mice. Thus far the γH tumors utilize only one of the two heavy chain alleles. Studies on the γA tumors have not been resolved as yet because the precipitating activity of the anti γA^{15} (C57BL derived γA) antiserum is not strong.

It is of interest that a protein carrying the unassigned-2 determinant has not yet been isolated from the series of tumors. While this suggests that the homologue of the (BALB/c) G protein will carry this determinant the possibility must be entertained that a completely different gene may exist on the C57BL/6 chromosome. In a study of the immunoglobulin types of wild mice from a number of U.S. sources and one Canadian locality, no mouse bearing the unassigned-2 determinant has been found. Mice imported from Kyushu, Japan, however, all carry the unassigned-2 locus. Thus it is a reasonable speculation that the heavy chain linkage group in C57BL/6 may be of Asiatic origin. Further, differences in natural populations carrying the -2 determinent and those with other characteristic markers e.g. G1, unassigned 3, 4 and 5 may be very great.

Other immunoglobulin heavy chain coisogenic lines are being developed from the heavy chain locus of AL/N, DBA/2 and NH.

Search for light chain alleles.

Most inbred strains of mice are homozygous for the genes that control only one of the 5 characteristic markers G1, unassigned 2, 3, 4 or 5. Among these strains light chain alleles have not been identified. Such loci are apparently quite rare. Thus it is possible that most inbred strains carry the same light chain locus. Possibly too most mice in the western hemisphere may carry the same light chain locus. It would be of great value to obtain alleles of light chains. The analysis of specific chains would aid in the study of the genetic control of immunoglobulin specificity, particularly in determining whether the light chains are controlled by two genes, one for the common region the other for the specificity region as proposed by Dreyer, Bennett and Hood. If a characteristic light chain marker located on the common region was

associated with different specificity regions particularly specificity regions containing deletions or insertions this would constitute proof of the 2-gene:1 polypeptide chain hypothesis. For this reason wild mice bearing the unassigned-2 heavy chain antigenic marker are likely candidates for carrying genetically different light chains. This presumption is based on the consideration that mice carrying the "2" are geographically separated. Another factor favoring the possibility of finding a light chain gene allele in Kyushu mice is that the separation of the progenitors of the BALB/c strain from wild Kyushu mice may have occurred a long time ago, thus providing ample time for mutations to occur and accumulate.

Mice from Kyushu bearing the unassigned-2 have been mated to BALB/c and plasma cell tumors will be induced in F_1 hybrids. Also the light chains will be isolated from these mice and amino acid sequence studies of the carboxyl and amino terminal residues will be studied in collaboration with Dr. Leroy Hood (C:I-5010).

Attempts to order the genes within the heavy chain linkage group have been unsuccessful. This has been attempted by seeking crossovers of the A and G genes. Two crosses have been intensively studied (BALB/c x C57BL/6)F_1 x C57BL/6 and (BALB/c x AL/N)F_1 x AL/N. No crossovers have been detected from over 500 mice in each of the two crosses. Further attempts to order genes in the immunoglobulin heavy chain locus have been hampered by the failure to link the heavy chain linkage group to other known genes in the 19 known (chromosomal) linkage groups. We are now completing the study of all the major linkage groups within the heavy chain loci.

Evidence that crossing over can occur has been obtained from studies on wild mice. Wild mice have been trapped in Maryland, Virginia and North Carolina and colonies have been established from wild mice imported from Japan and Taiwan. Unusual combinations of heavy chain markers have been found. All inbred strains carry only one of the 5 characteristic heavy chain markers. F_1 hybrids carry 2, but none carry more than 2. We have identified wild mice with 3 markers suggesting these are presumptive heterozygotes and that 2 markers are located on one chromosome.

From Kitty Hawk, North Carolina a mouse carrying the 3 unassigned markers 3, 4 and 5 has been trapped. This mouse has been mated to another mouse of the genotype G1/5. Five offsprings were obtained with the phenotypes: 3, 5; 3, 5; G1, 3, 4; 3, 4, 5 and G1, 3, 5. These progeny have been mated and studied further. Other mice from Kitty Hawk with phenotypes 3, 5 have been mated to C57BL 2/2 and all progeny are 2, 3, 5. Currently the evidence suggests 2 segregating loci.

One mouse from Kyushu has the genotype G1, 2, 4. This mouse has been successfully mated. The assignment of determinants to specific heavy chains and the recovery of gene products from these loci will provide detailed information on the comparative structure of the immunoglobulin heavy chain linkage group.

B. Specificity differentiation

Experiments have been performed over the last 5 years to determine if myeloma proteins have "antibody" or specific combining ability with known antigens. In these experiments antigens were introduced during plasma cell tumor induction and the myelomas produced were examined for ability to combine with the antigen. An experiment of this type in collaboration with Dr. E. Bruce Merchant (I:LI-151) is nearing completion. In this experiment red cells from 5 different species were introduced into BALB/c and the resulting tumors were examined by the Jerne plaque method and the Sterzl facilitation modification for ability to produce antibody that could attach to the various red blood cell surfaces; thus far none have been found.

While attending the Cold Spring Harbor Symposium Dr. Herman Eisen (Washington University School of Medicine) proposed that he screen the myeloma proteins for anti DNP activity, using the characteristic red spectral shifts produced with 2,4 dinitrophenyl groups. The initial survey of some 30 proteins produced several suggestive proteins. In the meantime, Dr. Eisen had sent a preparation of DNP-BGG to me and it was found that one of the proteins MOPC 315 strongly precipitated DNP and TNP substituted proteins in double diffusion. Since this time 116 proteins have been screened for DNP and TNP combining affinity. Two proteins MOPC 378 and MOPC 292 precipitated with TNP substituted proteins. The strong precipitating activity of 315 was confirmed by Eisen and almost immediatedly an intensive study of the 315 protein was begun. Dr. Henry Metzger (A:A&R-8c) of NIAMD (with whom I collaborated in 1966 on a for myeloma proteins with DNP activity) was involved from the beginning with Dr. Eisen and myself and he has begun a study of MOPC 315.

The MOPC 315 is a γA myeloma protein and is polydisperse having a variety of forms including components with sedimentation coefficients of 7, 9, 11, 13 and 15S. The polymeric forms possess the precipitating activity which is lost when the interchain disulfide bonds are reduced (with dithiothreitol) and S-carboxymethylated with iodoacetamide. The 4-chain unit, however, combines as avidly with DNP-substituted proteins, and further the papain Fab fragment combines with DNP-substituted proteins. Studies in progress include characterization of the subunits of the MOPC 315, crystallization of the protein and the initiation of an effort to amino acid sequence the 315 protein. Eisen will probably begin amino acid sequence studies of 315 this year; we will probably sequence a second IgA myeloma.

The screening of additional tumors is most desirable since other related IgA proteins will be of great value in determining the nature of the combining site. One other myeloma protein has been identified MOPC 329 and the two myeloma proteins 378 and 292 are being studied by hapten inhibition methods to search for better haptens.

The MOPC 378 is of unusual interest since it is a cryoglobulin - the first cryoglobulin thus far found in the mouse. Human 7S cryoglobulins have a pro-

pensity to crystallize. The MOPC 378 already forms small crystals in vitro, (too small for X-ray diffraction work). Work is in progress to gently reduce 378 (also a polydisperse γA) to a 7S form without destroying its cryo properties.

In collaboration with Dr. Myron Leon (St. Lukes Hospital) we have screened the myelomas in our collection for their ability to combine with Pneumococcus polysaccharides and Dextran. Two IgA tumors MOPC 167 and Mc 603 precipitate Type I, II and VI pneumococcus polysaccharides. Mc 603 precipitates in addition type XIV. Papain Fab fragment from 167 which is incapable of precipitating type VI pneumococcus polysaccharide inhibits the precipitation reaction indicating the combining activity is located on the Fab fragment. An anti-dextran antibody the IgM MOPC 104 protein precipitates dextran.

In collaboration with Dr. Leon we are seeking to identify the appropriate oligosaccharide ligands. At this time only preliminary statements can be made about the meaning of these findings to the specificity differentiation. Essential the preponderance of IgA myelomas in our series indicates a remarkable favoring of the IgA class differentiation. This group of antibody forming cells may be restricted in total number of specificities and our sampling may reflect the common types.

Clearly though the results indicate some myeloma proteins, e.g. MOPC 315, 378, 292, 603, 167 and 104 are normal proteins and that the clonalization of neoplastic plasma cells mimics a normal developmental process.

II. Protein synthesis studies

Studies in protein synthesis are being done collaboratively with Drs. J. Frederic Mushinski (C:LBgy-3222) and Phillip Periman (C:LBgy-3244). It has been hypothesized that one of the possible means for producing specificity variations in immunoglobulins through the availability of different t-RNA molecules. That is a single genetic message or codon can be translated alternatively depending upon the availability of different t-RNA's that identify the codon. An approach to this problem is to study the t-RNA profiles of plasma cell tumors. Dr. Mushinski has established the very complicated techniques for this analysis and has observed reproducible differences in leucyl t-RNA among several tumors examined.

A second approach is through the study of cell fusion. Dr. Periman has established plasma cell tumors in culture and is attempting to fuse these cells to determine if 'hybrid' proteins are synthesized as a result of fusion.

III. Studies on plasma cell tumor induction

In collaboration with Dr. Paul Anderson (C:LBgy-3240) studies in plasma cell tumor induction are being extended. Chemically defined components

of mineral oil are being tested. If successful these agents can be radio-labelled and used in tracing the fate of the oil.

Second a pathological comparison of oil and solid plastic plasma cell tumor formation is in progress.

Serial No. NCI-3202

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

Hudson, D. M., Finlayson, J. S., and Potter, M.: Linkage of one component of the major urinary protein complex of mice to the brown coat color locus. Genetic Res. Camb. 10: 195-198, 1967.

Coleman, T. J., Marshall, R. C., and Potter, M.: Preparation of a glycopeptide from an immunoglobulin kappa polypeptide chain from the mouse. Biochim. Biophys. Acta 147: 396-398, 1967.

Potter, M., and Lieberman, R.: Genetic studies on the immunoglobulins of mice. Cold Spring Harbor Symp. Quant. Biol. 32: 187-202, 1967.

Lieberman, R., Mushinski, J. F., and Potter, M.: Two chain immunoglobulin A molecules: Abnormal or normal intermediates in synthesis. Science 159: 1355-1357, 1968.

Potter, M.: The plasma cell tumors and myeloma proteins of mice In Methods in Cancer Research 2: Chapter 4, 105-157, Academic Press, New York, 1967

Potter, M., and Lieberman, R.: Genetics of immunoglobulins in the mouse. Advances in Immunology 7: 91-145, Academic Press, New York, 1967.

Smith, A. M., Potter, M., and Merchant, E. B.: Antibody forming cells in the pronephros of the teleost Lepomis Macrochirus. J. Immunology 99: 876-882, 1967.

Serial No. NCI-3209
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pathogenesis and pathophysiology of plasma cell neoplasms in mice and characterization of their protein products

Previous Serial Number: SAME

Principal Investigator: K. Robert McIntire, M.D.

Other Investigators:
Dr. L. W. Law (C:LBgy-3200)
Dr. N. Burstein (C:LBgy-3230)
Dr. E. Appella (C:LBgy-3229)
Dr. R. Rask-Nielsen (Copenhagen, Denmark)
Dr. P. Ebbesen (Copenhagen, Denmark)
Dr. W. Hall (C:LBgy-3225)
Dr. R. Asofsky (I:LGAR-116c)

Cooperating Units: Institute of Biochemistry A, University of Copenhagen
NIAID:LGAR(116c)

Man Years: Total - 2
Professional - 1
Other - 1

Project Description:

1. Plasma cell carcinogenesis

There are obvious advantages to studying carcinogenesis in the plasma cell system: The cells are highly differentiated and functional in that they produce and secrete a homogeneous immunoglobulin which serves as an extracellular marker for any given tumor. The problems of cellular differentiation and the genetic control of immunoglobulin synthesis can both be approached through plasma cell carcinogenesis and therefore the relationship of both of these to the neoplastic process.

Studies from this laboratory in germfree (GF) mice have shown a greatly reduced incidence of plasma cell tumors in GF BALB/c mice injected with mineral oil when compared with conventional (CVN) BALB/c mice. The incidence in GF mice was 5% while in ex-GF BALB/c mice from the same colony which were removed from the GF environment at weaning age the incidence was 80%. The latent period was also longer in GF mice, being 18 months compared to an average 12-13 months in CVN mice. The suggestion from this work was that environmental factors were of considerable importance in plasma cell carcinogenesis, along with the inducing agent mineral oil. To test the possibility that other agents

are necessary in the formation of plasma cell neoplasms the experiment of plasma cell carcinogenesis in GF mice has been repeated with the additional injection of aqueous subcellular filtrates from a CVN plasma cell tumor using 2 different tumors in 2 groups of mice. In addition the question of inheritance of specific immunoglobulin class and type is being studied by also injecting a RNA DNA extract of the same 2 tumors at the same time. This experiment is still in progress and results are not yet known.

Continued experiments with Drs. Ebbesen and Rask-Nielsen (University of Copenhagen, Denmark) in Copenhagen have shown that while extracts (subcellular centrifugates) of plasma cell leukemias and reticulum cell sarcoma, type B do produce a great increase in the incidence of plasma cell and reticulum cell neoplasia in BALB/c mice the same is not true in DBA/2 mice and in Wistar rats. These studies have been accepted for publication this year.

Induction of plasma cell tumors in SJL/J mice which have a high spontaneous incidence of reticulum cell sarcoma, type B has been tried by the injection of mineral oil intraperitoneally and this has been unsuccessful. At present (BALB/c x SJL/J)F_1 hybrids are being tested for plasma cell tumor (PCT) induction with mineral oil. Since the SJL/J mice have a tendency to reticular hyperplasia and the BALB/c to PCT induction there may be a possibility of getting more primitive forms of immunoglobulin producing cells to become neoplastic and therefore offer systems for studying the differentiation of the immune system. Work is also continuing on the study of delayed hypersensitivity in SJL/J mice. Initial results with intradermal testing with human serum albumin indicated that those mice with SJL/J disease (reticular neoplasia) had a diminished delayed hypersensitivity response.

2. Studies on mouse macroglobulins (γM)

Further studies on the tryptic digestion fingerprints of γM heavy chain (μ) indicate there is considerably greater variation in the pattern of μ chain than there is in heavy chains of other immunoglobulin (Ig) classes. Attempts are presently being made to split the μ chains with papain or trypsin to identify the location of the variation, whether it is in the carboxyl-terminal (Fc) piece or in the amino-terminal (Fd) piece as it is in other Ig molecules.

In collaboration with Dr. W. Hall (C:LBgy-3225) attempts have been made to negative stain γM molecules from PCT and also the γM of normal serum. These techniques have not yet been conquered, and we have not been able to compare the fixed structure of a wide variety of γM molecules. If this becomes possible it will be an excellent opportunity for studying the gross or tertiary structure of γM immunoglobulin, especially since one of the PCT products has been shown to have antibody activity against dextran.

There has recently been discovered by Dr. M. Potter (C:LBgy-3202) a PCT producing a γM in a BALB/c mouse which is coisogenic with C57BL/6 at the γG

locus. This is currently being used to aid in the search for subclasses in mouse γM and hopefully the discovery of allotypes which would make possible genetic testing of mouse γM: The demonstration of individual genes and their linkage or lack of linkage with other Ig genes.

As mentioned above, during the past year, one out of 8 γM mouse myeloma proteins has been shown to have the ability to specifically precipitate dextran. This activity is present in the serum and also in the purified γM, but not in the papain digestion fragments. Experiments are underway to study the role of the specific heavy (H) and light (L) chains when recombined with non-specific H and L chains, the importance of the polymeric structure of γM, the nature of the carbohydrate acting as ligand and the H chain sequence involved with the antibody binding site. Dr. Appella (C:LBgy-3229) is already in progress on the amino acid sequence of the L chain.

3. Light chain studies

The demonstration last year of apparent identity between two completely different tumor-produced λ type L chains raised the speculation that there is a finite number of genes controlling light chain synthesis or that there is a greatly limited variability in λ chains compared to κ chains. Very recently I have received a λ-chain producing tumor from Dr. Melvin Cohn (The Salk Institute) and this will be studied for comparison with the 2 previous λ chains.

In a study of the past year the ratio of κ and λ chains in normal mouse populations was determined. The measurement was made difficult by the fact that H and L chains must be separated before the L chains could be identified. In 4 different strains: BALB/c, C57BL/6, AL/N and DBA/2 the level of λ chains was less then 1% of the total light chains in both 7S and 19S immunoglobulins. This would explain the relative scarcity of λ-chain producing PCT.

4. Tumor antigens

In collaboration with Dr. N. Burstein (C:LBgy-3230) work has continued on the study of antigenicity of MCA induced fibrosarcomas in GF and CVN BALB/c mice. Thus far the results are suggestive but not clear-cut in favor of a diminished antigenicity in GF tumors when compared to CVN tumors. The majority of the work has been in the development and evolution of techniques which can be used in a quantitative manner. The immunization technique involves growth of the tumor in the foot for 2 weeks followed by amputation. Two weeks later the mice are challenged with a measured cell dose IV and the lungs are examined after 2 weeks for the presence of peripheral tumor nodules. The experiment is now awaiting the last repeat, using the latest evolution of the technique.

5. Urethan carcinogenesis

The incidence of urethan induced pulmonary adenomas is reportedly

increased following neonatal thymectomy in Swiss mice. This mimics the findings in several viral induced tumors in otherwise resistant mouse strains. Together with Dr. N. Burstein, we have tested GF BALB/c mice, which are free of the common viral infections, for the induction of lung tumors following IP urethan. The GF mice had 9/17 or 53% with tumors while 47/55 or 85% of the CVN mice had tumors when autopsied at the end of 20 weeks. This suggests that other agents, particularly those causing lung irritation, play a role in urethan pulmonary carcinogenesis. Experiments are now in progress to test for different pathways in GF compared to CVN mice in the metabolism of urethan.

Serial No. NCI-3209

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

Appella, E., McIntire, K. R., and Perham, R. N.: Lambda Bence Jones proteins of the mouse: Chemical and immunological characterization. J. Mol. Biol. 27: 391-393, 1967.

McIntire, K. R., and Law, L. W.: Abnormal serum immunoglobulins occurring with reticular neoplasms in an inbred strain of mouse, J. Nat. Cancer Inst. 39: 1197-1211, 1967.

Serial No. NCI-3218
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on interaction of tumor-inducing viruses and their host

Previous Serial Number: Same

Principal Investigator: Dr. Robert C. Ting

Other Investigators: Dr. L. W. Law (C:LBgy-3200)
Dr. M. Stanton (C:LP-516c)
Dr. K. Takemoto (I:LBV-83)

Man Years: Total - 2½
Professional - 1
Others - 1½

Project Description:

I. Studies on murine sarcoma virus, Moloney isolate [MSV (MLV)].

A. Biological and serological properties of a host variant of murine sarcoma virus. The viral particles present in a non-producer rat neoplasm (MSB-1) induced by murine sarcoma virus, as detected by electron microscopy was now found to be biological active on normal kidney cells of random bred Osborne-Mendel rats. The particles were found originally not infectious when mouse embryo cells were used as test cells. The virus is designated here as MSV (0). MSV (0) differs from other pseudotypes of MSV in its host range, antigenicity and interference pattern. Work is now in progress to investigate whether MSV (0) still retains its oncogenic potential, that is its ability to induce neoplasm in susceptible strains of rats.

B. Early detection of leukemogenic viruses in mice by their ability of rescuing MSV genome in MSB-1 cells. In collaboration with Dr. L. W. Law (C:LBgy-3200) work has been continued of studying in vivo rescue of MSV gemome of MSB-1 cells by various leukemogenic viruses. We have shown previously that superinfection of mixed culture of MSB-1 cells and mouse embryo (ME) cells with leukemia viruses resulted in the production of MSV which can easily be assayed as focus-forming unit(FFU) on ME cells. Experiments were designed to detect leukemia viruses in mice by their ability to rescue sarcoma genome from MSB-1 cells which have been inoculated into mice at various ages. The evidence for the presence of rescued MSV was determined by two criteria 1) histological evidence of the tumor growth showing typical MSV reaction 2) test tumor-inducing ability of the extract of the tumor in newborn Swiss mice.

Results showed that sarcomas were developed at the site of inoculation in mice infected with Moloney leukemia virus or Rauscher leukemia virus and challenged with MSB-1 cells as late as 21 days. These tumors showed typical MSV histological and MSV was recovered from tumor extract. Although control mice inoculated with MSB-1 cells often showed tumor growth at the site of inoculation, these tumors were typical of MSB-1 cells and contained no active MSV. By this technique we have also found that leukemia viruses in C58, AKR, C57BL/Ka mice could be detected at an early age. This technique should be useful to detect natural occurences of leukemia viruses in mice.

C. Studies on MSV infection in mice. In collaboration with Dr. L. W. Law (C:LBgy-3200) and Dr. M. Stanton (C:LP-516c), studies were carried out on biological, immunological and morphological effects in mice following infection with a murine sarcoma virus. It is found that a variety of neoplasm was induced in mice following infection with MSV. Adult mice were generally resistant to tumor induction by MSV, however, thymectomy would render the mice susceptible. Transplantation resistance could be readily demonstrated in adult mice infected with MSV or MLV and the mechanism of this rejection is apparently due to humoral antibody.

D. A quantitative in vitro assay for murine leukemia viruses by its ability to rescue MSV genome in MSB-1 cells. Work has been continued of developing a quantitative assay for murine leukemia virus. The ability of leukemia virus to rescue sarcoma genome in MSB-1 cells has now made it possible to assay leukemia virus quantitatively by its rescuing activity (RA). This assay is 5X more sensitive then complement-fixing (CF) tests developed by Hartley, et. al. The complete assay takes less than 21 days and the results obtained do not depend on other reagents such as complement and specific antisera which are often difficult to obtain.

II. Tumor induction in inbred strains of hamsters by various oncogenic viruses and properties of induced tumors.

The inbred strain hamster PD_4 is used to test their susceptibility to oncogenic viruses such as polyoma, SV40, adeno-12 and MSV. We have now obtained transplantable tumors by polyoma, SV40 and MSV. Both the polyoma tumor and SV40 tumor contain tumor specific T antigens as demonstrated by the immunofluorence. All tumors have the virus-induced tumor specific transplantation antigens.

III. In vitro transformation of inbred strain mouse embryo cells by SV40 virus.

In collaboration with Dr. K. Takemoto (I:LBV-83) attempt was made to transform a established cell line of AL/N mouse by SV40. Transformed cells contained tumor specific T antigen as demonstrated by immunofluorence. Tumor growth was obtained in AL/N mice, thymectomized at 3 days age and then received

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1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

300r X-ray irradiation at 4 weeks of age, with transformed cells but not with control uninfected cells. Transplantation antigens were also demonstrated with the transformed cells.

Serial No. NCI-3218

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

Ting, R. C., and Law, L. W.: Thymic function and carcinogenesis. Prog. Exp. Tumor Res. 9: 166-191, 1967.

Law, L. W., Ting, R. C., and Leckband, E.: Prevention of virus-induced neoplasms in mice through passive transfer of immunity by sensitized syngeneic lymphoid cells. Proc. Nat. Acad. Sci. 57: 1068-1075, 1967.

Ting, R. C.: Tumor induction in thymectomized rats by murine sarcoma virus (Moloney) and properties of the induced virus-free tumor cells. Proc. Soc. Exp. Biol. and Med. 126: 778-781, 1967.

Ting, R. C.: Failure to induce transplantation resistance against polyoma tumor cells with syngeneic embryonic tissues. Nature 217: 858-859, 1968.

Law, L. W., Ting, R. C., and Stanton, M. F.: Some biological, immunogenic and morphologic effects in mice following infection with a murine sarcoma virus I. Biologic and immunogenic studies. J. Nat. Cancer Inst. (in press), 1968.

Stanton, M. F., Law, L. W., and Ting, R. C.: Some biological, immunogenic and morphologic effects in mice following infection with a murine sarcoma virus II. Morphologic studies. J. Nat. Cancer Inst. (in press), 1968.

Ting, R. C.: Biological and serological properties of viral particles from non-producer rat neoplasm induced by murine sarcoma virus (Moloney). J. Virology (in press), 1968.

Serial No. NCI-3222
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Genetic and biochemical mechanisms controlling protein synthesis in well-differentiated neoplastic cells, mouse plasma-cell tumors

Previous Serial Number: SAME

Principal Investigator: J. Frederic Mushinski, M.D.

Other Investigators: Dr. M. Potter (C:LBgy-3202)
Miss R. Lieberman (I:LCI-15c)
Dr. J. Finlayson (BS-LBBP-43)
Dr. G. von Ehrenstein, Johns Hopkins Medical Center
Dr. M. Scharff, Albert Einstein Medical Center

Cooperating Units: NIAID:LCI
BS-LBBP
Johns Hopkins Medical Center, Baltimore
Albert Einstein Medical Center, New York

Man Years: Professional - 1

Project Description:

A. γA Myeloma Proteins

Work has continued on the characterization of the γA class of mouse myeloma proteins which is the type of myeloma protein produced by a preponderance of tumors in every series of mouse plasmacytomas.

Two different forms of γA myeloma proteins have been found. The most common form in our collection resembles the γA immunoglobulins described in other species including man, rabbit and horse. This type of protein can be isolated from mouse serum and ascites. The other form of γA protein has not been described in other species. It can be found in serum, but it characteristically is excreted in the urine of mice bearing the appropriate tumors.

Our interest has been to study both types of γA immunoglobulins and to compare them from a number of approaches.

Electrophoretically both proteins migrate in the β_1-γ_1 region on agar gel. Ultracentrifugation of the serum γA proteins characteristically reveals saw-toothed pattern of components with sedimentation coefficients clustering at 6.5, 9, 11, 13 S. Different peaks predominate in different proteins, and a

shift of predominance from lower to higher sedimentating components during storage in solution is seen. All the faster sedimenting species can be converted to a single 6.5S peak by reducing agents, and we are led to believe that the multiple forms are various polymers of a basic 6.5S molecule. The urinary protein, on the other hand, exhibits a major component sedimenting at 3.6S and only a very minor peak at 5.3S. The molecular weight of the 3.6S material appears to be about 65,000 which would be consistent with one light chain linked to one heavy chain, i.e. a γA half molecule.

These two forms of γA molecules have been shown to have different serologic specificities when studied with isologous antisera in Miss Rose Lieberman's (I:LCI-15c) laboratory. Further, reactions of partial identity were found with some antisera indicating extra antigenic determinants on the serum γA polymers. Tryptic peptide maps of isolated heavy chains from both γA types showed many peptide spots in common among all γA molecules, certain ones unique for each individual myeloma protein, and 5 spots present in both polymer heavy chains and absent in all 6 half molecule heavy chains. These spots, localized in tryptic peptide maps of papain Fc fragments of γA polymers seem to represent those areas of polypeptide chains found only in the polymers which lend them their antigenic determinants missing in half molecules.

Molecular weight determinations for the above heavy chains has been frought with difficulties but a 45,000 molecular weight for the half molecule heavy chain seems likely and is consistent with the 65,000 MW for the complete half molecule. Sephadex column chromatography in 6M Urea has confirmed our suspicion that the γA polymer heavy chain is significantly larger than that of the half molecule heavy chain. Electrophoresis in SDS-Urea-Polyacrylamide Gel is in progress for a similar comparison.

Studies are continuing in collaboration with Dr. Matthew Scharff (Albert Einstein Medical Center) into the sequence of assembly of the half and polymerizing molecules.

Studies are beginning to characterize the polymer-specific tryptic peptides in several such γA polymers. Since these peptides appear in the Fc fragment, the carboxyl-terminal portions of both heavy chains will be studied.

B. Translational Mechanisms Operating in Plasma Cell Protein Synthesis

To investigate the possibility of translational control playing an inportant part in determining immunoglobulin variability we have been isolating and studying transfer RNA (tRNA) from mouse plasma cell tumors, primarily those producing only kappa-type (κ) light chains.

A comparison of the chromatographically separable subspecies of various tRNA's specific for several amino acids was described last year utilizing Methylated Albumin Kieselguhr (MAK) columns and a reversed-phase ion exchange

column. Still better resolution results have been achieved with a reversed-phase ion exchange column described by Weiss and Kelmers utilizing Freon 214 as nonaqueous phase. Repeated detailed studies of the subspecies of tRNA specific for L-leucine (tRNA leu) have been performed on four κ chain-producing myelomas, normal mouse liver and one γA polymer-producing tumor. At least four subspecies of tRNA leu can be discerned using this column, and all four are seen in all tumors and tissues examined so far. A significant variation in the relative amounts of each tRNA leu component was seen among all the tumors, but no variation between different tRNA preparations from the same tumor line even when isolated six months apart and 12 transplant generations later.

Whereas this data does not contribute much to our question about the role of tRNA in controlling immunoglobulin structure, it does indicate that different tumors have different degrees of transcription of the DNA specific for each tRNA leu component. Thus these genes are operating independent of one another rather than simultaneously, and whatever controls the DNA transcribing activity for tRNA, it operates at a level finer than one turning on or off all tRNA leu.

Further investigations will expand this work to include 3 more κ chain myelomas, other tumor types and other normal tissues. Other amino acids to be studied will be lysine, arginine, serine and methionine.

Direct test of the translational control hypothesis will be tested by trying to change the nature of protein synthesized by subcellular components of myeloma cells in vitro.

Experiments done in collaboration with Dr. Gunter von Ehrenstein (Johns Hopkins Medical Center) and his colleagues have demonstrated the ability of tRNA leu from mouse plasma-cell tumors to insert leucine into rabbit hemoglobin in a reticulocyte ribosome in vitro system. No coding ambiguities were found when we compared the tRNA leu from two κ chain tumors or from one such tumor and normal mouse liver.

C. Computer Applications

Analysis of the column chromatographic data involves a time-consuming and arduous statistical analysis of two-isotope data from a liquid scintillation counter. To simplify and automate this process we had our counter equipped with a punched paper tape output facility. With the help of Dr. F. K. Millar (C:LPgy-944b) we developed a computer (IBM-1620) program suitable for handling this data once our paper tape (ASCII coded) was converted to another such tape with the proper BCD code.

To further automate this analysis process and,more significantly, to permit quantitative estimates of the amounts of tRNA leu present in each component

of the incompletely separated column eluate, an IBM-360 program was developed. With the help of Mr. John E. Fletcher (CR:LAS-3.10) the double-label data, this time in the form of punched cards, was reduced and analyzed as above. In addition, a subprogram supplying model curves of summed normal (Gaussian) distributions was added to closely approximate the real data with models formed from normal curves with known parameters and relative areas. Further, a subroutine for plotting the data and model curve was developed for use with the California Computer Products Plotter.

D. Other Work in Progress

1. Mouse Urinary Protein

In collaboration with Dr. John Finlayson (BS:LBBP-43) studies were furthered into the nature of the normal complex of proteins (MUP) found in mice. The three components of MUP were studied by tryptic peptide mapping and immunoelectrophoresis. Significant differences were found in peptide maps of MUP components I and III and on immunoelectrophoresis. Components I and II, on the other hand, are known to be controlled by a single genetic locus (MUP-a) and showed no differences on immunoelectrophoresis. A single extra peptide spot was consistently recovered from component I but never from component II.

2. Tissue Culture Transformation

In collaboration with the laboratory of Dr. Gunter von Ehrenstein, DNA isolated from κ chain mouse plasma-cell tumors was incubated with tissue culture cells derived from a plasma-cell tumor no longer secreting protein. Initial results indicating the successful induction of protein secretion are currently being followed up.

3. Clotting Factor X

In collaboration with Dr. David L. Aronson (BS:LBBP-43) a comparison of purified Factor X and prothrombin was made utilizing tryptic peptide mapping techniques. Resulting maps were so different that the possibility of these two components being the same is virtually ruled out.

4. In collaboration with Dr. John S. Finlayson, three components separated from purified human plasma β_2-Glycoprotein I were compared. No discernable differences among these three components and purified β_2-Glycoprotein I from Behringwerke AG were detected using ultracentrifugation, double gel diffusion (Ouchterlony), sialic acid analysis and tryptic peptide mapping.

5. In collaboration with Dr. Phillip Periman (C:LBgy-3244) metabolism and kinetics of protein synthesis are being studied in plasmacytomas and tissue cultures thereof, especially in those cells known to make γA myeloma proteins with antibody activity.

Serial No. NCI-3222

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

Finlayson, J. S., and Mushinski, J. F.: Separation of subfractions of human β_2-glycoprotein I. Biochim. Biophys. Acta 147: 413-420, 1967

Lieberman, R., Mushinski, J. F., and Potter, M.: Two-chain immunoglobulin A molecules: Abnormal or normal intermediates in synthesis. Science 159: 1355-1359, 1968.

Serial No. NCI-3229
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Chemical structure of immunoglobulin molecules

Previous Serial Number: SAME

Principal Investigator: Ettore Appella, M.D.

Other Investigators: Dr. Richard Perham, University of Cambridge, England
Dr. Ralph Reisfeld (I:LI-160)
Dr. R. T. Seki, Visiting Scientist, NIAMD

Cooperating Units: I:LI-160
NIAMD-LMB
University of Cambridge, England

Man Years: Professional - 1

Project Description:

I. Mouse light chains

A. Kappa chains

Kappa urinary proteins were produced by plasma cell tumors induced in BALB/c mice. A collection of about 100 proteins is now available for chemical characterization. These proteins are each single homogeneous entities having approximately 214 residues with a molecular weight of about 22,000 to 23,000. The data accumulated from a number of investigations (Hilschman and Craig, 1965; Titani, Whitley, Avogardo and Putnam, 1965; Perham, Appella and Potter, 1966; Gray, Dreyer and Hood, 1967) have clearly established that the amino-terminal half of these chains is extremely variable, the variations occurring in clusters of some one to five amino acids distributed along its approximately 107 residue length. The carboxyl-terminal half, however, so far is invariant. Sequence studies have been attempted by different approaches. S-carboxymethylated or S-amino-ethylated derivatives were subjected to enzymatic digest (tryptic, peptic or chymotryptic) and the hydrolyzates were applied to a Dowex 1 or DEAE-Sephadex column. The mixture of peptides was separated into ten to fifteen pools, each of which was subsequently fractionated on Dowex 50 or paper. Peptides in a yield ranging from 3% to 60% were obtained in high purity suitable for sequence studies. In order to derive a partial sequence cleavage with cyanogen bromide has also proved to be of great value.

Our studies have been focused first on the amino-terminal peptide. A comparison with other kappa chains has revealed that single amino-terminal

peptides of 24 residues could be isolated, indicating that in these proteins position 18 no longer occupied by an arginine residue as in MOPC-41A.

One striking result from our experiments is the isolation of identical amino-terminal tryptic peptides for two pairs of Bence Jones proteins (MOPC-31B and 178, MOPC-46 and 172). This observation has been further confirmed by the isolation of chymotryptic peptides on Dowex 50 for the two pairs of kappa chains. A large degree of identity is apparent in the elution diagram. Further work is in progress to establish the exact sequence of each variable region and define possibly a minimal difference in these proteins.

The mechanism for the sequence variability of kappa light chains is still unknown. From the data so far accumulated frequently only two alternative residues are observed at a number of positions. For example position 3 and 4 are occupied by valine or glutamine and leucine or methionine respectively. Other residues appear to be invariant; for example residue 2 in all kappa chains examined is isoleucine. Other positions, residue 18, may be hypervariable, more than two alternative amino acids are found. A unified hypothesis of antibody formation has been recently presented (Cold Spring Harbor Symp., 1967) whereby recombination between two cistrons is used to generate antibody variability (Smithies, 1967). Positions of hypervariability in light chains might then reflect genetic polymorphism. It is hoped that continuing work with the myeloma proteins of the inbred BALB/c mice, in which such polymorphism should be minimized, will substantially help in proving or disproving this and other hypothesis (Dreyer and Bennet, 1966; Edelman and Gally, 1967; Potter, Appella and Geisser, 1966).

B. Lambda chains

A lambda type of mouse myeloma light chain has been described for the BALB/c strain (McIntire, Asofsky, Potter and Kuff, 1965). This chain has a molecular weight of about 23,000 and is rarely selected in comparison to the kappa chain. Two such chains (MOPC-104 and RPC-20) have been recently analyzed for some of their chemical and immunological characteristics. The amino acid sequence of the carboxyl-terminal portion and amino-terminal portion has been determined following the isolation of tryptic, peptic and subtilisin-peptides. They are identical. Tryptic peptide maps prepared after complete aminoethylation of the chain, were found to be identical both for the arginine-positive peptides and ninhydrin-positive peptides. Continuing investigation of the amino acid sequence is now in progress and should reveal whether these two chains are in fact identical. The lack of sequence variations could be the result either of myelomatosis or of a marked constraint on the expression of the lambda chain gene in the mouse. One possible mechanism whereby the mouse lambda chain could be constrained with respect to variation is by translocation of this gene to another part of the genome such that it is not subjected to that process, whatsoever it might be, that ordinarily generates antibody variability.

II. Rabbit light chain

There are six allotypic specificities in the rabbit distributed in two series a and b consisting of three alleles each (Dudin, 1960). The b group is carried by the light chain, the a group by the heavy chain. We have been interested in analyzing the carboxyl-terminal portion of the three allotypic products of the light chains. The first group b4 has been carefully studied. From the peptides derived from peptic and tryptic digestion of the performic acid oxidized light chain, a partial amino acid sequence has been deduced as follows: Ser-Phe-Asn-Arg-Gly-Asn-Cys. Comparison of the carboxyl-terminal sequences of mouse, human and rabbit light chains shows a striking homology. This similarity indicates a common genetic origin and may well be of critical significance for the specific association of light and heavy chains through the interchain disulfide band. This sequence also indicates that the b4 light chain by homology belongs to the kappa type already described for humans and mice.

The amino-terminal sequence of rabbit light chains of the b4 group has been reported to be Ala-Val-Val-Val-Gln-Gln (Doolittle, 1966). A remarkable homology between this sequence and the mouse lambda chain can be obtained if one assumes a deletion of the N-terminal residue in the rabbit chain.

Ala-Val-Val-Val-Gln-Gln Rabbit chain

Glp-Ala-Val-Val-Thr-Gln-Gln Mouse chain

This deletion will correspond to pyrrolidone carboxylic acid, the amino terminal residue ordinarily found with lambda chains and heavy chains and occasionally with kappa chains. The existence of an enzyme aminopeptidase-type capable of removal of the amino-terminal pyrrolidone carboxylic acid can be postulated in analogy to a bacterial system where such peptidase has been already described and partially purified (Doolittle, 1968).

Work in a number of laboratories has suggested that a population of light chains devoid of allotypic specificities controlled by the genes at the b locus is produced in normal rabbits (b-negative). Such chains have not been separated from mixtures of normal light chains. However, it has been possible to suppress the phenotypic expression of b5 allotypic specificity by administration of anti-b5 to developing newborn b5 homozygous rabbits (Dubiski, 1967). The IgG produced by the treated animals was then essentially all b-negative for several months.

Structural studies of the carboxyl and amino-terminal regions of these b-negative chains has revealed considerable homology with myeloma lambda chains of man and mouse. In both man and mouse kappa and lambda chains are antigenically distinct. In an analogous manner the b-negative and b-positive rabbit chains can also be differentiated by a specific antiserum. A remarkable

finding is the observation that a relationship of the b-negative light chains to another locus c7 (P) determined by an allele at a locus different from a and b could be demonstrated (Mage, unpublished). A test for genetic linkage of the b-positive and b-negative light chains thus become possible for the first time by using these antigenic markers.

Serial No. NCI-3229

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

Appella, E., and Perham, R. N.: The chemical structure of immunoglobulin light chains. Cold Spring Harbor Symp. 32: 70, 1967.

Appella, E., and Perham, R. N.: The amino terminal sequences of two mouse lambda chains. J. Mol. Biol. 33: 1968.

Appella, E., Mage, R. G., Dubiski, S., and Reisfeld, R. A.: Chemical and immunochemical evidence for different classes of rabbit light polypeptide chains. Proc. Nat. Acad. Sci., (in press), 1968.

Serial No. NCI - 3230
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A study of urethane carcinogenesis

Previous Serial Number: Same

Principal Investigator: Nelson A. Burstein, M.D.

Other Investigators: Dr. K. R. McIntire (NCL:LBgy-3209)
Dr. A. C. Allison, Mill Hill, London
Dr. W. Hall (NCI:LBgy-3225)

Man Years: Professional - 1

Project Description:

I. Urethane tumors in germfree mice

Urethane produces lung tumors in mice. Thymectomy increases the incidence of urethane-induced adenomas (alveologenic carcinomas.) Since thymectomy increases the incidence of certain virus-induced tumors, an infectious agent might be involved in urethane carcinogenesis. Since germfree mice are free of bacteria and common viruses, we gave urethane to germfree and conventionally maintained mice. The germfree BALB/c mice developed fewer tumors (9/17) than did conventional mice (47/55) (<0.025). Electron micrographs were made by Dr. W. Hall (C:LBgy-3225). This revealed no differences between germfree and conventional tumors.

All animals were tested for the following viruses: PVM, Reo-3, GD VII, K, Polyoma, Sendai, MVM, M-adeno, M. Hepatitis, LCM. No specific virus was associated with pulmonary tumors. Two tumor bearing germfree animals had HI activity against MVM. In the conventional mice there was no correlation between HI activity to MVM and tumors. All conventional mice, normal and tumor bearing, treated or control, had HI activity against PVM or Sendai. Since these viruses produce an inflammatory and proliferative response in the lungs of mice, they might be augmenting the effect of urethane. This work was done in collaboration with Dr. K. R. McIntire (C:LBgy-3209) and Dr. A. Allison, Mill Hill, London.

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1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

II. Metabolism of urethane

Since germfree mice might metabolize urethane differently than conventional mice, we (Dr. K. McIntire, C:LBgy-3209) modified Rogers' methods to show that there are no gross defects in urethane metabolism in germfree mice.

III. Cell free filtrate of conventional adenoma

Such a filtrate, given to germfree mice, will not restore a normal incidence of urethane-induced tumors.

IV. Lung tumors in conventionalized germfree mice

Germfree mice are conventionalized at weaning, maintained 4 to 6 weeks in an animal room, and treated with urethane. They develop more tumors than germfree mice and fewer than conventional mice. Work is in progress (with McIntire, C:LBgy-3209) to foster nurse germfree mice on conventional mothers and vice versa. These mice will be treated with urethane and the tumor incidence compared with conventional mice.

V. Lung tumors in vitro

Embryo lung has been placed in organ culture and treated with urethane and metabolites of urethane. At first there is disorganization in cultures treated with urethane or its metabolites. Alveolar proliferation develops only in those cultures treated with a metabolite of urethane. Work is in progress comparing the histology of these transformed cultures with the pulmonary tumors obtained *in vivo*. Work is in progress to isolate and test the metabolite in tissue culture.

Serial No. NCI-3240
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Pathogenesis of murine plasmacytomas

Previous Serial Number: NONE

Principal Investigator: Paul N. Anderson, M.D.

Other Investigator: Dr. Michael Potter (C:LBgy-3202)

Man Years: Professional - 1

Project Description:

A. Histogenesis of murine plasma cell tumors induced by mineral oil or plexiglas discs:

1. The histogenesis of mineral oil-induced plasmacytomas in the BALB/c mouse has been studied by Potter and MacCardle (J. Nat. Cancer Inst. 33: 437, 1964). Merwin and co-workers (Proc. Soc. Exp. Biol. Med. 101: 437, 1959; J. Nat. Cancer Inst. 31: 997, 1963) have reported studies of plasmacytoma development in mice bearing diffusion chambers or components of the chambers, including a limited number of tumors following intraperitoneal implantation of plastic discs. In anticipation that comparison of the mineral oil and disc induction of tumors might further clarify the origin of the neoplastic plasma cell, morphological studies of tissues of BALB/c mice with intraperitoneal implantation of the two materials are underway.

Approximately two hundred mice have received three intraperitoneal injections of Baylor F (0.5 cc per dose, 3 doses two months apart), and are being examined at frequent intervals from day one throughout the entire period of tumorigenesis. Fifty mice have had 24 mm diameter plexiglas discs inserted intraperitoneally, and tumors have begun to appear after about 4½ months.

Histological examination of tissues during the period of plasma cell tumorigenesis includes standard histochemical stains, such as haematoxylin and eosin, thionin, methyl green-pyronin, and acridine orange. Ultrastructure of the progressive cellular reactions involved is being followed by electron microscopy in collaboration with Dr. Nelson Wivel (NCI-4833). In anticipation of obtaining radioactively-labelled pure hydrocarbons (see below), autoradiography is planned for addition to histological examinations of these tissues.

With the desire to extend these examinations of plasma cells, two weeks was spent in the laboratory of Dr. John J. Cebra (Biology Department, The Johns Hopkins University, Baltimore) learning techniques of fluorescent labelling of

of proteins and of specific fluorescence immunohistochemistry. Using fluorescence microscopy, after staining tissues with specific antisera to defined mouse immunoglobulins (antisera labelled with fluorescein isothiocyanate), cells containing specific immunoglobulins can be located normal lymphoid tissues, plasmacytomas, and developing oil granuloma and fibrous tissue during tumorigenesis. Particular effort has been made testing a variety of fixatives, to provide preparations with both preservation of adequate antigenicity for fluorescence immunohistochemistry of high specificity and high quality morphological preservation--cold ethanol has proved most satisfactory to date, but further fixative trials (hydroxy-adipaldehyde, freeze-drying, freeze-substitution) are planned. Studies are in progress comparing "producing" and "non-producing" (of myeloma protein) tumors with histochemistry, electron microscopy and fluorescence immunohistochemistry. Although results to date indicate that a high degree of success with fluorescence immunohistochemistry can be anticipated in the murine plasmacytoma systems, technical problems with fixation (minor) and presently inadequate equipment for fluorescence microscopy hinder the present realization of the potential inherent with this technique.

2. Mineral oil comprises a poorly-defined mixture of saturated hydrocarbons. Due to its poor definition, specific testing and specific chemical modification, as well as strict knowledge of the chemical species involved in plasma cell tumor induction, are virtually impossible. Efforts are being made to find and test substitutes for mineral oil which might provide a single compound (or compounds) of high purity for inducing BALB/c plasmacytomas and/or immunological adjuvants, with desired emulsifying properties, but which additionally could be chemically defined and modified. Experiments are underway to determine pathogenicity, tumor incidence, cellular response and adjuvant activity of (a) purified hydrocarbons of the types found in mineral oils (straight and branched-chain paraffinic alkanes and branched cycloparaffinic condensed saturated-ring compounds) and (b) high and low viscosity dimethyl silicone fluids.

Initial studies (Potter) showed previously that a straight-chain alkane, n-hexadecane, has too high a short-term toxicity in mice for general use. Three non-branched cycloparaffins (cyclohexane, decahydronaphthalene and perhydrophenanthrene) caused 100% rapid fatalities in mice in present experiments. Studies with relatively impure (as commerically available) hexamethylnonane, tetramethylpentadecane and squalane, and highly purified 7-n-hexyloctadecane, have been initiated by intraperitoneal injections of these materials into groups of BALB/c mice.

Sources for adequate amounts of pure 7-n-hexyloctadecane and a pure branched cycloparaffin, to be tested for plasmacytoma induction, are being sought. These last two compounds are also being sought as ^{14}C-labelled compounds for body distribution and metabolism studies in collaboration with Dr. John Boitnott and Dr. Simeon Margolis of the Johns Hopkins University School of Medicine, Baltimore. These two latter compounds will also be used for

synthesis of dinitrophenyl-addition compounds, for use in attempts to induce plasmacytomas which might produce myeloma globulins with antibody activity against the dinitrophenyl moiety, a few of such tumors having been found by Dr. Potter (C:LBgy-3202) in mineral oil induced plasmacytomas.

3. In collaboration with Drs. Boitnott and Margolis, studies are in progress comparing tissue levels of saturated hydrocarbons in tissues of mice of strains BALB/cAnN, C3H/HEN and C57BL/6N (which have marked differences in susceptibility to plasmacytoma formation in response to mineral oils), after intraperitoneal injections and short or long-term oral feeding of low and high viscosity mineral oils, using organic analytic techniques developed in their laboratory. If radioactively-labelled pure hydrocarbons can be obtained, further metabolic studies of saturated hydrobarcons in mammals are planned.

4. Short and long-term experiments are underway feeding high and low viscosity mineral oils to BALB/c mice to determine if plasmacytomas can be induced by the oral route in this strain which is very susceptible to plasma cell tumorigenesis following parenteral injections of mineral oils.

B. Study of specific immunoglobulin antibody forming cells in vitro:

Plasma cell tumors producing IgA with antibody activity to dinitrophenyl-, trinitrophenyl-, and Pneumococcal polysaccharide groups have been demonstrated by Dr. Potter in BALB/c mice. This phenomenon provides an opportunity to determine the relative frequency of cells secreting anti-DNP, anti-TNP, or anti-polysaccharide antibody in normal tissues, as well as in lymphoid tissues of mice immunized with these immunogenic materials, in oil granuloma or disc-induce fibrous reactive tissues, and in plasmacytomas. Studies are being planned, using a modification of the Sterzl-Jerne plaque-forming technique to demonstrate frequency and distribution of such antibody-secreting cells. In this technique, dispersed cells from the various tissues to be tested are plated over agar mixed with red blood cells which have been chemically coupled to DNP, TNP, or pneumococcal polysaccharide. Complement is added after specific rabbit anti-mouse IgA serum has been incubated with the plates, and single antibody-secreting cells will be localized by the production of lysis and plaque formation. Further definition of such antibody-secreting cells will include fluorescence immunohistochemistry and electron microscopy.

C. Studies of cellular differentiation in embryonic development in Ilyanassa obsoleta:

A culture of the marine gastropod, Ilyanassa obsoleta, has been established, and studies related to the influence of the polar lobe of the early blastomeres of this species (a remarkable example of cytoplasmic segregation in the early cleavage stages) have been started. In cooperation with Dr. Roger Ladda, of the Armed Forces Institute of Pathology, electron micrographs of early stages have shown a remarkable relationship of the nuclear membrane to the formation of annulate lamallae and cortical granules, and

further ultrastructural studies of this embryo are planned. Studies using radioactive labels and polar lobe-specific fluorescent-labelled antisera are planned to trace distribution of polar lobe materials in subsequent development, using radioautographic and fluorescence immunohistochemical techniques. Metabolic studies using radioactive tracers, using biochemical techniques in collaboration with Dr. Ladda and Dr. Joran Piatigorsky (NICHD).

Publications: None

Serial No. NCI - 3241
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study on virus-host cell interaction and subsequent detection of related viral antigens with emphasis on murine sarcoma-leukemia complex

Previous Serial Number: Same

Principal Investigator: Duc Duc-Nuigeen, M.D.

Other Investigators: None

Cooperating Units: Dr. Janet W. Hartley (NIAID:LVD)

Man Years: Total 1 1/4
Professional - 1
Other - 1/4

Project Description:

Studies on antigens related to a murine leukemia virus (Rauscher strain) propagated in established rat kidney cells. To determine whether more than one viral antigens related to Rauscher virus are detectable in rat kidney cells infected with this virus. Recent studies have shown that when complement fixation tested, sera obtained from Osborne-Mendel rats carrying Rauscher lympho-sarcomatous thymus reacted more strongly with Rauscher antigens derived from rat kidney cells than with those derived from mouse embryo fibroblasts. This finding suggested that more than one complement fixing antigens were present in rat kidney cell (NCI-NR) infected with Rauscher virus. Experiments are being carried out to determine whether the same observation can be made in different strains of rats. These studies offer a better understanding of the various virus-related antigens in neoplastic cells.

In vitro study of cellular adsorption of a murine sarcoma virus (strain Harvey). To find a way of increasing the infecting particles/input multiplicity ratio in cell-murine sarcoma

(or leukemia) virus system. Different compounds are being tested for their enhancing or reducing effect on the adsorption of murine leukemia and sarcoma viruses to host cells. Certain polycations were found to have this enhancing effect and the efficiency of adsorption could be increased more than 20 fold. Work is being carried out to determine what viral components of the leukemia-sarcoma viral complex is affected by the enhancing compounds. These studies on the adsorption efficiency of murine sarcoma or leukemia viruses will undoubtedly further clarify the concept of a "helper virus" in viral oncology.

Study on the mechanism of viral hemadsorption. To learn more on the binding force, the binding site and the surface changes of virus-infected cells in the hemadsorption phenomenon. The concomitant observation of membrane-free aggregates of viral nucleocapsid in the cytoplasm and attached red blood cells on the surface of the same cell infected with mumps virus indicated that only infected cells hemadsorbed and that hemagglutinum is confined within the infected cell. The attachment of red blood cells to morphologically intact cell membrane prior to its differentiation into viral envelope suggested the hemadsorption phenomenon dependent on the presence of hemagglutinin, was independent of the viral maturation process. The study on viral hemadsorption offers a better understanding on the interaction between two cells carrying related antigenic components. Some sort of modification in the technique might make the hemadsorption phenomenon applicable to studies on oncogenic viruses.

Efforts are being made to obtain virus-free tumors in laboratory animals inoculated with Harvey virus. The induction of tumors in hamster, mouse and rat by a murine sarcoma virus (Harvey strain) was successfully carried out in our laboratory. Several attempts to obtain cell lines derived from these tumors have failed. However we have had no difficulty in establishing transplantable tumors when the original tumor was induced by Harvey virus-transformed cells.

In the coming fiscal year (1969) these same studies on Rauscher virus and Harvey virus will be continued and expanded. The investigator will have better electron microscopic facilities thus enabling him to use the immuno-ferritin approach to Rauscher leukemia and Harvey sarcoma viruses.

Serial No. NCI - 3241

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

Duc-Nguyen, H.: Hemadsorption of mumps virus as examined by light and electron microscopy. J. of Virology, 2: May, 1968. (In press).

Duc-Nguyen, H.: Enhancing effect by DEAE-dextran on the focus forming titer of a murine sarcoma virus (Harvey strain). J. of Virology, 2: June, 1968. (In press).

Serial No. NCI-3242
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Fluorescent antibody studies of and purification of polyoma, SJL and murine sarcoma viruses.

Previous Serial Number: None

Principal Investigator: Robertson Parkman, M.D.

Other Investigators: Dr. R. C. Ting (C:LBgy-3218)
Dr. L. W. Law (C:LBgy-3200)
Dr. S. Strober (C:LBgy-3243)

Man Years: Professional - 1

Project Description:

1. Fluorescent Antibody Studies

Fluorescent antibody studies have been carried out with Dr. R. C. Ting (C:LBgy-3218) to investigate the replication of murine sarcoma virus (MSV) in tissue culture.

These studies have necessitated the purification of MSV as an antigen. Commercially available fluorescent antibody was unacceptable; and, therefore, fluorescent rabbit anti-rat serum was prepared. It is planned to study the various pseudotypes of MSV using this technique and to correlate these findings with neutralization and cytotoxic studies.

In conjunction with Dr. S. Strober (C:LBgy-3243) studies to determine the site and time course of antibody production in normal and thoracic duct depleated rats have been started. In depleated animals after tetanus toxoid stimulation there is no antibody production in the spleen while most of the production appears in the regional nodes.

2. Neonatal Studies

The passive presence of hemagglutinin inhibiting antibody (HI) in a mouse will protect the mouse from infection by polyoma virus as demonstrated by a lack of tumor immunity upon subsequent polyoma tumor challenge.

Newborn mice receive passively HI antibody via the colostrum of polyoma infected mothers. Peak antibody titers are reached at 8-12 days and are no longer detectable at 35-45 days after birth. Newborn mice of normal and infected mothers have been injected with polyoma virus at 0, 8, 35, 45 and 60

days after birth. One month later they are challenged with polyoma tumor cells. The absence of tumor growth is taken as evidence of tumor immunity and thus successful prior polyoma infection. Animals nursed by infected mothers showed no tumor immunity if they were infected with polyoma virus at a time when they had HI titers. However, some animals nursed by infected mothers no longer had demonstratable HI titers were resistant to polyoma virus infection at 35-45 days after birth as shown by susceptibility to tumor challenge. This resistance may be due to nondetectable amounts of antibody or to other passively acquired factors. Further studies to determine among these and other possibilities have been undertaken with Dr. R. C. Ting (C:LBgy-3218).

3. Noninfective Polyoma Studies

The presence of HI antibody has been correlated with tumor immunity. However, since viral multiplication may be necessary to produce tumor immunity, it was decided to attempt to separate the production of HI antibody from the production of tumor immunity. For this purpose the separation of infective polyoma virus (containing DNA) and noninfective polyoma virus (containing only the protein coat) by $CsCl_2$ density gradient has been undertaken. Once a suitable separation has been achieved, mice will be challenged with both preparations and assayed for HI activity. Then they will be challenged with polyoma tumor cells to determine their state of tumor immunity.

4. SJL Disease

With Dr. L. W. Law (C:LBgy-3200), attempts to purify a virus from the reticuloendothermal tumors of SJL mice have been undertaken. After density gradient purification, appropriate fractions were injected into newborn SJL and BALB/c mice which are now under observation.

Serial No. NCI-3243
1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the initiation of primary immune responses by circulating and noncirculating lymphocytes in the rat.

Previous Serial Number: None

Principal Investigator: Samuel Strober, M.D.

Other Investigator: L. W. Law (C:LBgy-3200)

Man Years: Professional - 1

Project Description:

1. The role of circulating lymphocytes in transplantation immunity has been carefully studied. Several investigators have shown that these cells can initiate graft vs. host disease and the homograft reaction. On the other hand, the role of circulating lymphocytes in the initiation of primary humoral antibody responses is not clear. There is some evidence that these cells initiate the primary hemolysin response to sheep erythrocytes in the rat, however, there are no reports of similar experimental work in other antigen-antibody systems.

The present investigation has been designed to test the ability of circulating lymphocytes to initiate primary antibody responses to several antigens; sheep erythrocytes, bovine serum albumin, diphtheria toxoid, tetanus toxoid and horse spleen ferritin. Attempts were made to restore the antibody response of sublethally irradiated rats to each antigen with thoracic duct cells or spleen cells taken from normal syngeneic donors.

Preliminary results indicate that circulating lymphocytes initiate the response to some antigens such as sheep erythrocytes and bovine serum albumin, but that noncirculating lymphocytes found in the spleen initiate the response to others, such as tetanus toxoid and diphtheria toxoid.

Further studies are being carried out to determine the functional and morphological features of the noncirculating lymphocytes and their relation to the thymus.

One corollary of this work is that cells which initiate the rejection of organ transplants may be different from the cells which initiate immunity to bacterial infections.

Serial No. NCI-3243

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Publications:

Strober, S., and Murray, J. E.: Studies of the enhancing properties of plasma of murine parabionts. Transplantation 15: 1371-1379, Nov. 1967.

Serial No. NCI - 3244

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on controls of protein synthesis: Mammalian cells.
A. Mouse plasma cell tumors, in vitro.
B. Antibody synthesis.
C. Cell fusion.

Previous Serial Number: None

Principal Investigator: Phillip Periman, M.D.

Other Investigators: Dr. Michael Potter (C:LBgy - 3202)
Dr. J. F. Mushinski (C:LBgy - 3222)
Dr. George Yerganian(Childrens Cancer Research Foundation, Boston)

Man Years: Professional - 1 (Dr. Periman)

Project Description:

The mouse plasma cell tumor system is an excellent one in which to study protein synthesis in mammalian cells. These tumors secrete distinct proteins that are partial or complete immunoglobulins. Some of these have recently been shown to have antibody activity. These proteins can be characterized chemically and immunologically. Each tumor secretes a specific immunoglobulin and this does not change after years of repeated transplantation.

The advantages of having these plasma cell tumors in vitro have been recognized for some time. A homogeneous population of cells could be manipulated so as to observe the effects of antimetabolite and mutogenic agents on cellular patterns of nucleic acid and protein metabolism. Until recently no one had been able to grow these cells in tissue culture, although several competent investigators had tried. Pettengill and Sorenson were the first, and to this date, the only investigators to report any success culturing mouse plasma cell tumors. (Exp. Cell Research, 47: 608, 1967).

Serial No. NCI - 3244

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

In this laboratory, during the past year, we have been able to grow several of the tumors *in vitro*. All of them have been adapted to tissue culture by using a technique published by Sato (PNAS, 48: 1184, 1962). Solid tumors were minced into a cell suspension and cultured. After a few days, these cells were injected into BALB/c mice. The subsequently developing tumors were recultured. After a few days in culture, these cells were reinjected into a new host. This cycling of tumor cells from mouse to tissue culture was carried out until the cells grew readily *in vitro*. This usually required three to six passages. This procedure probably selects a population of cells that has metabolic or membrane characteristics that enable them to survive *in vitro*. By serial passage these cells became the predominant ones.

Using the techniques of double diffusion in agar (Ouchterlony) and radio-immunoelectrophoresis, these cells have been shown to continue to secrete the same immunoglobulin secreted *in vivo*. They also produce the same myeloma tumor when injected *in vivo*. Preliminary studies with fluorescent antibodies have shown labelling of these tissue culture cells.

We have been able to grow every tumor line which continued to cycle. In culture at the present time are MOPC 265, a Kappa chain only producer; AS, a nonproducer; MOPC 47T, a γH producer; and MOPC 315B, a γA producer. Other lines are in the process of being adapted.

When I came to this laboratory on July 1, 1967, there were no facilities available for this work. A great amount of my time has been spent assembling the equipment necessary to carry out the work. I am convinced that any line can be adapted to grow *in vitro* if the investigator is willing to expend the effort and constant attention which the adaptation of these tumors demands.

One major problem remains before this system is complete for experimental work. That is the development of clones. In all attempts to date, cell lines have died unless the population was over 10,000/ml. This minimal inoculum requirement has made it impossible at this time to clone these cells. However, we hope to use a soft agar technique to get them to grow in clones.

Serial No. NCI - 3244

1. Laboratory of Biology
2. Carcinogenesis Section
3. Bethesda, Md.

Recent work by Okada (Exp. Cell Research 26: 98, 1962), Harris, (J. Cell Science, 1: p.1, 1966) and Sorieul and Ephrussi, Nature, 190: 653, 1963) and their co-workers has shown that it is possible to fuse cells of different origins and species. These observations are exciting in that they open a new system for investigating the regulation of genes and gene action. I hope to be able to produce cell fusion between different plasma cell tumors. This will result in mixed cytoplasm which may result in inducers or repressors in one cell type to affect another. This mixing may result in a change in the type of immunoglobulin produced. In Kappa chain only producers a heavy chain locus may become activated and production of this new heavy chain detected. Heavy and light chains may mix resulting in immunoglobulin secretion completely different from those of the parent cells. If this happens, then quantitative analysis of the various combinations may yield data about the number of gene copies for each chain which are active.

One problem with the cell fusion experiments is the detection and isolation of the fused cells. One approach which I have just begun to use is to use histocompatible differences between the parent cells as a selecting mechanism. The data on this work is not complete.

Another approach is to mate cells of different species, then use chromosomal differences and hybrid vigor to select fused cells. To this end I have initiated a series of experiments with Dr. George Yerganian of the Childrens Cancer Research Foundation, Boston, using his line of inbred hamster cells.

For a long time it has been suspected that mouse myeloma tumors secrete immunoglobulins that functionally are normal; however, it has only been recently that antibody activity has been demonstrated. MOPC 315B which I have growing in vitro secretes a γA protein which reacts specifically with DNP or TNP protein conjugates. Using this tumor as a prototype, Dr. Fred Mushinski (C:LBgy-3222) and I have initiated a series of experiments on the assembly and secretion of antibody. We also hope to develop a cell-free system for protein synthesis using this tumor line. The appearance of antigen binding affinity should signal the completion of the active site on the immunoglobulin.

Publications: None.

Serial No. NCI-3204 (SAME)
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Genetics of cancer in mice

Principal Investigator: W. E. Heston

Other Investigators: George Vlahakis, with collaborative research with Drs. L. W. Law, William T. Hall, and M. Ben-David

Man Years:
Total: 4
Professional: 2
Other: 2

Project Description:

Objectives: The aim of this program is to gain further knowledge of the causation of cancer. Emphasis is placed on the genetic factors in the causation of cancer but these cannot be studied alone. Viral factors, particularly the mammary tumor virus, are studied in relation to the genetic factors. This year a study aimed at the effect of neonatal thymectomy on carcinogenesis has been completed.

Another general objective with this basic program in which we maintain many strains of mice with a number of viral-genetic combinations is to collaborate with younger investigators to help them get their research programs underway.

Methods: Three types of neoplasms including lung tumors, mammary tumors, and hepatomas in their respective mouse strains are studied. In the studies on the effect of the thymus in oncogenesis in collaboration with Dr. Law, strain C3HfB and C3HfB hybrid mice were used because the C3H females are such good mothers that they raise most of their thymectomized young. Thymectomy was done neonatally. The lung tumors were induced in thymectomized and intact C3HfB and (C3HfBxA)F_1 hybrids with repeated injections of urethan. Although we had previously found no effect of thymectomy on lung tumors induced with MCA, Trainin had reported an increase in urethan induced lung tumors with thymectomy and it was necessary to see if we could confirm his findings. The animals were killed at specific ages and the lung tumors were counted with the aid of the dissecting microscope.

In the study of the effect of thymectomy on hepatomas the hepatomas were allowed to arise spontaneously. Strain C3HfB mice and those of strain C3H-A^{vy}fB that are even more susceptible than the C3HfB were used. The mice

were killed at specific ages and examined for the occurrence of hepatomas.

Usual genetic breeding procedures are followed in the genetic analyses and in the development and maintenance of the various strains. Foster nursing is used in developing the lines without the mammary tumor virus and with specific combinations of genotype with the virus. Studies on the mammary tumor virus are being done in collaboration with Dr. Hall. Here the milk is used as the main source of agent and it is obtained readily with a simple hand milking device from lactating females after they have been injected with oxytocin.

Effect of hormonal agents in relation to the prolactin releaser, perphenazine, to the genotype of the animal and to the presence or absence of the mammary tumor virus in the development of the mammary gland has been studied in collaboration with Dr. Ben-David by using a number of our inbred strains and lines of each strain with or without the mammary tumor virus.

A rather extensive study of the premalignant hyperplastic nodules in the mammary gland of mice is underway. Three strains, C3H, DD, and BALB/c, and two lines of the mammary tumor virus, that from C3H and that from DD, are used in various strain-virus combinations. The glands are resected, cleared and stained by the technique devised by the Berkeley group and the nodules are located and counted under the dissecting microscope.

Results: The study of the effect of neonatal thymectomy on the induction of lung tumors with urethan and on the occurrence of spontaneous hepatomas has been completed and the data tabulated. Despite Trainin's reported observations, in our study thymectomy had no effect upon the induction of lung tumors. Response was measured by the average number of induced lung tumors at a given time. In the more resistant C3HfB thymectomized and control mice averages in the different groups ranged from 3.5 to 4.0 tumors per mouse, but there was no difference between the thymectomized and control groups. In the more susceptible (C3HfB x AfB)F_1 groups the average number of lung tumors ranged from 24.27 to 30.87 but again there was no difference between the thymectomized and control groups.

The hepatoma studies included thymectomized and control highly susceptible C3H-A^{vy}fB males killed at 9 and 12 months of age and females killed at 12 and 13 months of age. This gave a range of response from 29 percent to 100 percent but the thymectomized groups certainly did not give a greater response. If there was any difference the controls had a higher response. In thymectomized and control groups of the more resistant C3HfB males killed at 9 and 12 months and females killed at 14 months, the responses ranged from 33 to 62 percent, but again the thymectomized groups did not have a higher incidence of hepatomas. In fact, there were slightly higher incidences in the control groups.

These data and the earlier data on MCA induced lung tumors certainly show that thymectomy does not increase either lung tumors or hepatomas. It would appear that thymectomy has its effect on those tumors that are viral induced and that neither lung tumors or hepatomas in the mouse are viral induced.

An interesting observation in the C3HfB females injected with urethan was the induction of a few mammary tumors. This confirms Tannenbaum's observation that urethan is even carcinogenic in the induction of mammary tumors and extends his observations when the urethan was painted on the skin to showing the same effect when urethan is injected intraperitoneally.

Some of our most interesting observations in the mammary tumor problem is the development of mammary tumors in strain BALB/c. For decades BALB/c has been considered a very low mammary tumor strain without the mammary tumor virus but genetically susceptible to it. Recently Deringer observed that in her well cared for breeding colony mammary tumors did occur in 19% of the breeding females. We are finding mammary tumors in even a higher incidence of breeding females at an advanced age and when we examine them more closely we find even more with very small mammary tumors. Yet Dr. Hall can find no A or B particles in any of these tumors under the EM. Dr. Gil Smith likewise has examined a BALB/c mammary tumor and hyperplastic nodules and has found no particles. In other mammary tumor virus-free lines like C3HfB one does find B particles in the tumors which the Berkeley group have called nodule inducing virus (NIV) in contrast with the usual mammary tumor virus (MTV). Some of these old BALB/c mice do show nodules as well as the tumors. It would, therefore, seem that these genetically susceptible BALB/c females can develop nodules and tumors in the absence of either the NIV or the MTV when they have the hormonal stimulation from breeding over a very long period of time.

The study of the hyperplastic nodules of the mammary gland has revealed that the nodules cannot be considered solely due to the NIV any more than the tumors are due solely to the MTV, if indeed there is an NIV distinct from the MTV. Instead, both are due to a combination of genotype, line of virus, and the hormonal stimulation. (The effect of physical and chemical carcinogens was not included in this study but they, too, could have an influence.) Strain C3H females with a strong genetic influence and a potent virus have nodules by 3 months of age even when kept as virgins. Strain DD females with a weaker virus either qualitatively or quantitatively do not have nodules by 3 months when kept as virgins but do have them by 6 months. DDfC3H, that is, DD with the C3H line of virus, is intermediate between DD and C3H in that there are a very few nodules in the 3 month old virgins and more in the 6 month old group. DDfB females which according to the Berkeley group would have the NIV do not have nodules at either 3 or 6 months when kept as virgins, but older breeding DDfB females do have nodules. BALB/cfDD or BALB/c females with the DD line of virus do not have nodules at 3 months when kept as virgins but do have nodules by 6 months. Straight BALB/c females that apparently have neither MTV or NIV, as no particles have been observed with the EM, have no nodules when kept as virgins up through 12 months of age with the possible exception of one 9 month old female. However, we do observe nodules in BALB/c females that are bred by the time they are 9 months old.

In the study of the effect of perphenazine, a prolactin releaser, on mammary growth in various inbred strains with and without the mammary tumor virus in collaboration with Dr. Ben-David, it was found that the strains showed genetic differences in response to the perphenazine and also that in each case

the mammary tumor virus increased the growth of the mammary gland induced by the perphenazine. A more complete report will be given by Dr. Ben-David.

Significance of the Program to the Institute: These basic studies on mammary cancer in the mouse should have great significance in guiding our thinking about possible causes of tumors in other species including man. Although there is a virus or a complex of viruses involved, this tumor should not be considered a viral disease any more than it is a genetic disease, a hormonal disease, or a physical or chemical carcinogen disease. It is doubtful to me that a strictly viral approach will reveal a virus in the causation of breast cancer of women, but someone will have to come up with some new approach which will not only consider the possibility of a virus but of these other factors as well. Because of the complexity of genetics of the human population, such an approach will be difficult. We should not let up on the hormonal approach to breast cancer in women.

This program continues to be of general significance in that it continues to form a base from which younger investigators can build their research programs, using our basic information and material in the form of our well defined strains with their specific genotype-viral combinations.

Proposed Course of the Project: This program will continue along the lines of these basic studies with these three neoplasms of the mouse, lung tumors, hepatomas, and mammary gland tumors, with the mammary tumors receiving greatest emphasis. The immediate study with the hyperplastic nodules will have to be rounded out with observations on breeding females. These observations should then be correlated with observations made in collaboration with Dr. Hall of particles seen with the EM. Considerable emphasis will also be placed on the study of these particles in the milk of females of these various strain combinations and these observations can be correlated with tests for the virus by Moore with his micro-Ouchterlony double-gel diffusion test or the Melpar nodular test for the agent. With Dr. Hall we are interested in females that do and those that do not get mammary tumors within an inbred strain. Is it because some have the virus and others do not, or is there a quantitative difference in the virus?

Publications:

Heston, W. E. and Vlahakis, G.: C3H-A^{vy} - a high hepatoma and high mammary tumor strain of mice. J Nat Cancer Inst, June 1968.

Ben-David, M. and Heston, W. E.: Effect of perphenazine, a prolactin releaser, and estradiol on the mammogenesis of various strains of mice with and without the mammary tumor virus. Submitted to J Nat Cancer Inst

Serial No. NCI-3208 SAME
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The study of the inheritance of murine proteins and their relationship to the incidence of tumors.

Principal Investigator: Harold A. Hoffman

Other Investigators: Miloslav Rechcigl, Jr., with collaborative research with Herbert Abelson, Jan Moor-Jankowski, Melvin Reuber, and Gilbert Smith

Man Years:
Total: 4
Professional: 1
Other: 3

Project Description:

Objectives: To study by methods appropriate to biochemical and quantitative genetics the inheritance of proteins in the mouse and other mammals, and to determine the genetic and environmental relationships between the proteins and tumors. To determine the primary structure of the normal protein and abberant or mutant protein in order to define the gene or genes controlling their production and determine the time of gene action during the development of the animal.

Methods: In these investigations mice from 18 inbred strains and substrains, which represent at least 6 different lines of known origin, are used as sources of proteins and for the production if isologous antisera, New Zealand white rabbits of known immunoglobulin type are used for the production of heterologous antisera, and non-human primates of the order anthropoid used as a source of protein.

Immunochemical methods, agar-gel-double diffusion and immunoelectrophoresis, are used in the detection and characterization of different proteins. Physical and chemical techniques of starch-gel electrophoresis, agar-gel electrophoresis, block electrophoresis, and chromatography, both gel filtration and ion exchange, are used to isolate and classify different proteins. The find structure and minute differences between closely related proteins are determined by peptide map analysis (fingerprints) and amino acid analysis.

Results: Inheritance of murine urinary proteins. The electrophoretic phenotype on starch gel of urinary protein type 1a (3 bands) and 1b (4 bands) has been verified on agar-gel electrophoresis and acrylamide-gel electrophoresis. Both the starch-gel and acrylamide-gel electrophoretic systems are

run with a discontinuous buffer systems, Tris EDTA, pH 9.1, and borate buffer, pH 8.6, while the agar-gel electrophoresis is run with a continuous buffer system, Tris acetate, pH 5.5. This new finding indicates that the urinary protein complex is made up of 4 components which are not artifacts of the electrophoretic methodology. A second finding is that at different pH's the protein components have different electrophoretic mobilities. At pH 9.1 components 1, 2, 3, 4 are equivalent to components 3, 1, 2, 4 respectively, at pH 5.5 (components are designated in order of increasing anodic mobility).

A three-factor linkage test has been started to map the Up-1 gene on linkage group VIII. The two marker genes are brown (b) and Pin-tail (Pt).

Identification of murine viral agents. In collaboration with Drs. H. Abelson and G. Smith studies utilizing horseradish peroxidase as an electron-opaque marker for the ultrastructural localization of viral antigens were initiated. Lymphocytic choriomenigitis virus (LCM), Rauscher leukemia virus, and mammary tumor virus (MTV) are the systems being investigated. The long-range objective of this work is to examine murine models of carcinogenesis and autoimmune disease with respect to viral etiology. To date we have successfully conjugated the enzyme to the anti-LCM and anti-MTV antibody and have developed an in vitro assay by double-gel diffusion.

Erythrocyte catalase in inbred mice. The quantitative determination of erythrocyte catalase in 18 inbred strains and substrains has been completed. Genetic origin and sex were found to be the main two factors determining the level of enzyme activity. The NBL strain (an outcross from C57BL/10) was found to have the lowest level of catalase activity, 9 units/mlRBC/sec, while BALB/c was found to have the highest activity, 32 units/mlRBC/sec. These two strains are being crossed to produce F_1 and backcross generations to determine the inheritance of erythrocyte catalase activity.

Quantitative inheritance of pulmonary tumors in mice. The analysis of incidence of induced tumors in the F_1, F_2, and backcross generations of the A/He by C57BL/6 cross is in progress. Data are not presented at this time because the number of animals examined is small. The experiment to determine the route of administration of the MCA has been completed and the data are presented in Table 1. Treatment group A is being used in the genetic cross.

Proposed Course of Project: Emphasis will be placed on the molecular analysis of the urinary proteins. Peptide map analysis (fingerprints) and amino acid analysis will be used to characterize the 4 protein components of the urinary protein complex. Effort will also be directed toward isolating liver and erythrocyte catalase from C57BL/He, C57BL/6, and BALB/c to determine if two species of catalase exist as has recently been demonstrated in man.

Table 1. - Number Lung Tumors in A/He Strain Mice[1]

Treatment[2]	Sex	Number Examined[3]	Mean Number Nodules
A	♂♂	42	34.8
	♀♀	22	54.8
C	♂♂	37	0.4
	♀♀	29	0.2
D	♂♂	28	22.0
	♀♀	9	79.9
E	♂♂	33	1.4
	♀♀	9	0.2
B	♂♂	27	13.6
	♀♀	11	9.9
F	♂♂	21	185.5
	♀♀	4	229.8

(1) Mice were treated at 8 weeks of age.

(2) A: Single I.P. injection of 0.5 mg MCA in 0.5 ml incomplete adjuvant
B: Single I.P. injection of 0.5 mg MCA in 0.5 ml aqueous suspension
C: Single I.P. injection of 0.5 ml incomplete adjuvant
D: Single I.P. injection of 0.5 mg MCA in 0.5 ml complete adjuvant
E: Single I.P. injection of 0.5 ml complete adjuvant
F: Single I.V. injection of 0.5 mg in 0.5 ml aqueous suspension

(3) Examined 16 weeks after injection of MCA

Publications:

1. Hoffman, H. A. and A. J. Gottlieb: Hemoglobins of non-human primates. In Starck, D.,R. Schneider & H. Kuhn (Eds.): Progress in Primatology. Stuttgart, Gustav Fischer Verlag, 1967, pp. 417-421.

2. Peacock, A. C., Gelderman, A., Ragland, R., and Hoffman, H.: Haptoglobin levels in serum of various strains of mice. Science 158: 1703-1704,1967.

3. Hoffman, H. A. and A. J. Gottlieb: Hemoglobins of chimpanzees and gibbons. In Primates in Medicine, Basel/New York, Karger, 1968, Vol. I, pp. 27-34.

Serial No. NCI-3224 SAME
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Carcinogenesis of the liver in Buffalo female and male and ACI and Fischer female rats of varying ages given carbon tetrachloride.

Principal Investigator: Melvin D. Reuber

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: Study the susceptibility of female and male rats of different ages to carcinogenesis of the liver.

Methods: Buffalo female and male rats were 4 weeks, 12 weeks, 24 weeks, 52 weeks, or 76 weeks old at the start of the experiment. They received subcutaneous injections of carbon tetrachloride and corn oil for 12 weeks.

Major Findings: The 24 or 52 week old animals developed a higher incidence of hyperplastic lesions and more advanced hyperplastic lesions of the liver than did the younger animals. They also had a small number of early carcinomas. There were, however, fewer hyperplastic lesions in the liver of the 76 week-old animals.

Cholangiofibrosis developed in the livers along with hyperplastic parenchymal cells. Cholangiofibrosis was found in male rats more frequently than female rats. It was also present in the older animals more often than the younger animals. Even though there was a decrease in the hyperplastic lesions in the 76-week-old animals, the incidence of cholangiofibrosis was the same.

Significance: The development of carcinomas of the liver following the administration of carbon tetrachloride has not been described in the rat. The older animals are more susceptible to the induction of tumors than are the younger animals. With fluorenamine compounds the younger animals develop a higher incidence of carcinomas of the liver.

The cholangiofibrosis is similar to that seen in animals given other chemicals (p-dimethylanimoazobenzene, 3'-methyldimethylaminoazobenzene, N-2-fluorenyl- or N-2-fluorenyldi-acetamide, p-dimethylaminobenzene-1-azo-1-

naphthalene, N',N'-2,7-fluorenylenebisacetamide, N-hydroxy-N-fluroenylacetamide) and shown to be precarcinogenic.

Proposed Course: Carbon tetrachloride was administered to all age groups of male and female rats for a longer period of time in order for the animals to develop a higher incidence of carcinomas of the liver. The carcinomas were transplanted into isologous hosts.

The project was extended to include ACI male and female rats, and Fischer female rats.

The data have not been analyzed because we are awaiting the preparation of histologic sections.

Publications:

1. Reuber, M. D. and Glover, E. L.: Hyperplastic and early neoplastic lesions of the liver in Buffalo strain rats of various ages given subcutaneous carbon tetrachloride. J Nat Cancer Inst 38: 891-899, 1967.

2. Reuber, M. D. and Glover, E. L.: Cholangiofibrosis in the liver of Buffalo strain rats injected with carbon tetrachloride. Brit J Exper Pathology 48: 319-322, 1967

Serial No. NCI-3225 SAME
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Biophysical and bioassay studies on various murine and human pre- and post-neoplastic tissues in vivo and in vitro.

Principal Investigator: William T. Hall

Other Investigators: Collaborative research with Dr. Howard E. Bond, Dr. Virginia J. Evans (NCI-3213), Dr. Janet W. Hartley, Dr. Walter E. Heston (NCI-3204), Dr. Donald Morton, and Dr. Katherine K. Sanford (NCI-3213).

Man Years:
Total: 3
Professional: 1
Other: 2

Project Description:

Objectives: The object of this program is to investigate in vivo and in vitro on a broad spectrum in both pre- and post-neoplastic tissues the occurrence at an ultrastructural level of some recurrent peculiarity which could furnish a clue to the etiology and process of neoplastic transformation. In this laboratory the particular marker most frequently chosen has been a morphologically detectable virus. Thus, we have been generally concerned with two somewhat similar murine RNA viruses - mammary tumor virus and leukemia virus.

Methods: Our laboratory is especially concerned with biophysical techniques employing the electron microscope and ultracentrifuge. Several areas of research are involved and include: (1) development of methods for viral detection, isolation, and identification; (2) determination of the role in tumorigenesis of specific detected viruses; (3) refinement of techniques of viral quantitation and assay; (4) parameters of viral inhibition. To these ends we have surveyed with the electron microscope a large number of murine and human tumors and derived tissue cultures, as well as pellets from density gradients on which tumor and cell fractions had been placed. Various assays have been used to complement these investigations.

The Laboratory of Biology (NCI) maintains over thirty inbred strains of mice from among which substrains have been developed which demonstrate respectively wide variations (0-100%) in mammary tumor incidence. From several of these, milk and tumor samples have been collected and studied for the presence of oncogenic capabilities.

Results: That the murine mammary tumor virus (MTV) is a mammary tumor agent (MTA) is well established; that it is the necessary etiologic agent in the production of mouse mammary cancer is still doubtful. Several so-called MTV-free mouse strains have been reported in the literature, but when tumors do occur in these strains, MTV is generally detectable by electron microscopy (EM). Recently, however, with Dr. Walter E. Heston, we have been studying a substrain of 'MTV-free' BALB/c mice which now has a 40% tumor incidence. Approximately twelve tumors have been thoroughly studied with the electron microscope. Thus far no MTV has been found. We are now examining the milk of this substrain for the virus and applying quantitative bioassay studies.

In all of this work with MTV, one of the most critical problems has been the lack of a reliable short term quantitative virus assay. The available tests for the presence of virus and its nodulogenic potential require at best 4.5-5.0 months and the standard tumorigenic bioassay takes about two years. Moore and his co-workers are developing an Ouchterlony immunodiffusion test which requires only 0.04 cc. of material - tumor extract or milk - to test within two days for the presence of MTV. In collaboration with Dr. Moore (South Jersey Institute for Medical Research) and Dr. Walter E. Heston, we are attempting to determine the reliability and feasibility of this test.

In a preliminary experiment, milk samples from eleven mouse strains and substrains were tested and gave results consistent with what is known of the presence or absence of MTV in the respective colonies.

To determine the value of the test in predicting the proneness of specific females to develop mammary cancer, another study was undertaken. Forty strain A female mice (tumor incidence 45%) were bred for three litters. On the tenth and twelfth day of the second lactation, each animal was milked. One sample of milk was tested by the Ouchterlony method and the other sample sent to Melpar, Inc. for bioassay. This bioassay, sensitive at dilutions even greater than 10^{-6}, requires 24 weeks and is related to the production of hyperplastic alveolar nodules in the test animals. The animals from which the milk was taken are under observation for tumor development. Those which develop tumors will be compared with those which gave positive Ouchterlony and/or Melpar results. Thus far, 36 milk samples have been tested for the presence of MTV antibody: 16 were positive, 18 negative, and 2 very weakly positive.

The Ouchterlony test is admittedly not very sensitive and a negative response may result from a low titer rather than from a complete absence of virus antigen. In the production of mammary tumors by MTV, quantity of virus is undoubtedly significant, but there is also a question of virulence. In collaboration with Dr. William Feller (Georgetown University Medical School), we reported on a combined ultracentrifugation, EM and bioassay investigation of milk from several mouse strains. This study clearly demonstrated that the tumor inducing potency of virus from C3H mice and from the Andervont Wild mice was not exclusively a quantitative matter. Put into genetically identical hosts (BALB/c), all of which were similarly handled, the C3H-virus proved to be more tumorigenic and more rapidly so than its Wild counterpart; yet far more virus particles were present in milk of BALB/cf Wild than BALB/cfC3H.

The spontaneous occurrence of mammary tumors in the Andervont Wild mouse colony has been approximately 3%. Yet when BALB/c/An (1% with tumors at the average age of 22.0 months) are foster nursed on Wild mothers, the BALB/c tumor incidence rises to 71% at 15.9 months and virus is readily recoverable, suggesting that it was also present in the foster mothers. Thus far information about the presence of MTV in Wild mice has been mostly inferential. We have undertaken a direct study of these animals. Breeding pairs of agent-free BALB/c/He mice were foster nursed respectively on 6 Wild mothers and will be tested for the presence of virus in their milk at their first litter. Milk was also taken from these Wild mice for Moore's Ouchterlony test and the Melpar bioassay. Thus far, all Wild milks tested by Moore have been negative, which suggests at least three possibilities: (1) the MTV has been lost by the Wild mice during their confinement in the laboratory; (2) the MTV is present but in titer so low that it escapes detection by the Ouchterlony method; (3) the MTV is present in a form antigenically different from that for which the Moore test was designed.

The Melpar hyperplastic alveolar nodule test is more sensitive and may add more information. Ultimately, of course, the real proof that MTV is in the Wild mice must be virus recovery in a recognizable and infective form.

For this reason, with Dr. Howard E. Bond we have attempted to refine the existing MTV isolation techniques. The method most commonly used was developed by Moore and Lyons and consists in separation of MTV from milk or mammary tumor extract by a series of differential centrifugations followed by the suspension of a pellet on a density gradient. Because of the various pelleting steps and resultant clumping, much of the virus material is lost in preparation. We have devised a method in which EDTA-clarified skim milk is put directly on a sucrose gradient in a 650 cc. capacity zonal centrifuge rotor. In the original determination of virus distribution, thirty 20 cc. fractions were removed from the gradient, pelleted and examined by EM for the presence of MTV. In subsequent preparations fractions Nos. 19-27 (the MTV-containing zones) were removed and 9 cc. of each layered respectively on a 17 cc. 4-20% Ficoll-heavy water density gradient and centrifuged in a Spinco #30 fixed angle rotor. In each gradient tube a narrow sharp light scattering band appeared at density 1.16 and was shown by EM to be almost pure MTV. The method has been repeated several times with similar results and bioassays of the separated virus and other zones throughout the tubes are now in progress.

Since the process involves no pelleting, loss due to clumping or differential centrifugation is kept to a minimum and the entire procedure from fresh milk to purified virus can be run in about seven hours. Thus, not only is the normal method shortened, but the possibility of recovering virus from low titer samples is greatly enhanced.

Along with the genetic modes of resistance to murine mammary cancer, its viral etiology suggests, at least hypothetically, another possible mechanism of resistance - that of viral inhibition. Recently, in collaboration with Dr. Virginia Evans, we isolated a C-type virus (4705-virus) from a C3H minced mouse embryo tissue culture originated on completely chemically defined medium. By complement fixation and neutralization tests, the virus was shown to belong antigenically to the Gross leukemia virus group. Although able to

infect virus-free tissue cultures from various mouse strains, the virus was non-leukemogenic when 0.1 cc. of a gram equivalent was injected intraperitoneally or subcutaneously into newborn BALB/c, C3H/He, or DD mice. Squartini has already demonstrated the effect leukemia virus has in postponing the onset of mammary tumor formation. Since we had isolated an attenuated leukemia type virus, we attempted a similar study, with Dr. Walter E. Heston.

Into fifty newborn MTV-bearingC3H/He mice, 0.1 cc. of a gram equivalent of 4705-virus was injected. All the virus was from a single stock which had been taken from fibrosarcomas formed by implantation of the originally infected tissue culture into C3HfB/He MTV-free mice. Fifty animals were also retained as controls. As each tumor appeared, a piece was prepared for light microscopy and a piece for electron microscopy. From the remainder, gram equivalent microsomal fractions were prepared by homogenization and differential centrifugation. These were tested for the presence of MTV antigen (by Dr. Dan H. Moore) and leukemia virus antigen (by Dr. Janet W. Hartley) with the following results:

C3HfB/He mice	MTV Positive / No. Animals	Leukemia Virus Positive / No. Animals Tested
Control	41/41	2/41
MTV + Leukemia Virus	36/36	17/36

Six control extracts unequivocally negative for leukemia virus antigen and six with the highest leukemia antigen titer were selected from the test material. These have been injected into weanling BALB/c mice to test the inhibitory effect of the leukemia virus when it is introduced simultaneously with MTV into a virus-free mouse strain. When introduced into C3H/He subsequent to MTV infection, tumors arose*.

In the process of screening tissue cultures for virus contamination, four cell strains originated by cloning from one common cell of subcutaneous connective tissue origin were examined by EM and complement fixation techniques in collaboration with Dr. K. K. Sanford and Dr. J. W. Hartley. The particles encountered were of two main types: (1) a 100 mu particle which buds from the plasma membrane and which in the mature form resembles the characteristic C-type particle; (2) a 70 mu so-called A-type particle, which buds from intracytoplasmic membranes. The relative distribution of each particle type is shown below:

Comparison of concentration of A- and C-type particles (per thin section of 25 distinct cell sections), MuLV antigen and recovery of viruses of MuLV family from 4 mouse embryo tissue culture cell strains

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* approximately 3 weeks later than in control mice.

Cell Strain	Medium	CF Antigen		Average Number of Particles/Cell Section Direct Test		
		Direct	Passage	A	C-Budding	C-Mature
3069	NCTC 135 + 10% H.S.	-	+*	14.5	0.4	0.3
4953	NCTC 135	-	+	18.2	1.0	0.0
2472	NCTC 135 + 10% H.S.	+	+	2.5	0.7	11.0
4700	NCTC 135	-	-	16.3	0.2	0.5

*(+) indicates development of MuLV group reactive CF antigen in NIH-METC inoculated with cell suspensions of test lines, indicating replication of C-particle virus of the MuLV family.

By various procedures, the C-particle was shown to be infective in several mouse tissue culture systems and to function as a "helper" virus (by Dr. Robert Ting) in the recovery of Moloney sarcoma virus. Likewise, the characteristics of and relationship between A- and C-type particles were investigated. Cell extracts were passaged onto virus-free Swiss and C3Hf mouse embryo tissue cultures; CF and EM tests were again made to investigate the infective capacities of the two particle types. These data are summarized in the following table:

Comparison of relative concentrations of A- and C-type particles, MuLV CF antigen, and recovery of viruses of the MuLV family from 4 cloned mouse embryo tissue culture cell lines.

Cell Strain	CF-Ag* Direct Test	Relative** Concentration Particles(EM)	Passage in Swiss Mouse Embryo Cells						Passages on C3H Embryo Cells	
			First		Second		Third			
			CF Ag.	EM Part Conc.	CF Ag.	EM Part Conc.	CF Ag.	EM Part Conc.	CF Ag.	EM Part Conc.
3069	> 16	$C = C_B > A$	16	C, C_B	16	C, C_B	8	C, C_B	2	C, c_B
4953	< 2	$A > c, c_B$	<2	-	<2	-	<2	-	<2	-
2472	> 16	$C > C_B$ a	8	$C_B > C$	16	C, C_B	NT		4	C, C_B
4700	(2)	$A > c_B$	<2	-	<2	-	<2	-	<2	-

* Reciprocal of CF antigen titer in sonicated cell packs; parenthesis indicates partial reactivity.

** A = intracisternal A-particle, C = C-particle, C_B = budding C-particle. Lower case = very small number of particles; NT = no test done.

Although C-particles were found in cultures exposed to extracts from the C-particle-bearing strains, no A-particles were found in any passage culture. These results indicate that the intracisternal A-particle was neither infective nor developmentally associated with the C-particle. A positive CF test was correlated with the concentration of morphologically detectable C-particles and independent of the presence of A-particles.

Besides the research on murine tumor-tissue culture systems, similar work is now in progress in collaboration with Dr. Donald Morton on human osteogenic and lipo-sarcomas and derived tissue cultures. Although this work is still in its preliminary stages, several very interesting features have already emerged, which encourage continued intensive investigation.

Significance to the Program of the Institute: In mouse mammary adenocarcinoma, we have one of the relatively few authenticated cases of virus intimately associated with the appearance of malignant tumors. The tumor-associated virus exists also in the milk and thus has easy access to all the offspring. Although it is generally conceded that the mammary tumor virus is the mammary tumor agent, it is, at the same time, known that the mammary tumor agent is not the sole carcinogenic influence in this system. Greater clarification of the precise role of the virus in the induction of neoplasia, be it independent or complementary, would be an important contribution to our knowledge of the etiology of viral carcinogenesis. This is especially true in view of the human breast cancer task force which is supporting large scale investigations into human mammary carcinogenesis. Part of this program includes screening tumors and milk for the presence of virus, precisely paralleling work already done in mice. In the murine MTV study, we thus have at hand a model system about which much is known and which could very ably contribute to a deeper understanding of the human problem.

On the one hand, the development of a repeatable method for separating out and identifying very small quantities of virus would provide a most useful tool to those presently searching for virus by EM scanning procedures with their inherent sampling limitations. Likewise, the presence of a sensitive immunological test for virus and the added detection of proneness to tumor formation would give invaluable prognostic assistance to the physician. On the other hand, the clear-cut demonstration of spontaneous virus-free mammary tumors would add another dimension to the search for "the" etiologic agent. Ultimately, for information about human virus-caused disease, research must inevitably return to viruses as they exist in randomly breeding populations, the wild type, away from the unreal world of the laboratory controlled inbred strains.

The intracytoplasmic, intracisternal A-particle has been reported in several cell types, especially in plasma cell tumors, and some have speculated that it represents the precursor of the leukemia virus. Knowledge of its nature and function is certainly relevant to any continued study of viral oncology and/or carcinogenesis.

Proposed Course of the Project: Since becoming project officer of an NIH contract awarded to Melpar, Inc. and designed to carry out MTV bioassays, we have expanded our efforts to develop a reliable short-term bioassay by using the contract facilities. Two large scale statistical bioassays are in progress which will bring accurate quantitation to an MTV bioassay for the first time. Likewise, it will provide a known standard reference to which all subsequent tested unknowns can be referred. Among the materials to be tested are:

(1) "Agent-free" BALB/c tumor extracts and milk, in search of a mammary tumor line which is truly lacking the mammary tumor agent;

(2) Milk from Wild and BALB/cfWi mice for presence of a wild-type virus;

(3) Fractions from C3H milk separated by our zonal-density gradient technique to see if we have a consistently repeatable band of high infectivity;

(4) Milk from A mice which has previously been tested by Moore's Ouchterlony method. Hopefully this will indicate the extent of the reliability of Moore's test and measure its worth as a prognostic device.

At least one more experiment must be done to determine the inhibiting effect of 4705-virus on MTV. Quantities of both viruses will be separated on gradients and then with the MTV concentration kept constant, various amounts of 4705 will be added to experimental animals which will then be observed for the occurrence of hyperplastic alveolar nodules and tumors.

Of great importance to our program is a further refinement of the MTV separation and bioassay using ultracentrifugation and the Melpar contract. If a reliable methodology is developed, then specific gradient fractions can be bioassayed, even though virus titer may be too low for actual visualization of light scattering bands. This, obviously, has great application to the human mammary tumor problem.

Most of our research efforts will be spent on the human tumors which we have recently acquired. Initially the tumors, tumor-derived tissue cultures and developed plaques will be studied with the electron microscope. At the same time, chromosome analyses of the tissue cultures will be made.

Publications:

1. Hall, W. T., Hartley, J. W., Andresen, W. F., Sanford, K. K., and Evans, V. J.: Virus particles and murine leukemia virus complement-fixing antigen in neoplastic and non-neoplastic cell lines. Science 156: 85-88, April 1967.

2. Hall, W. T. and Sanford, K. K.: Virus particles in eight murine cell strains of single cell origin. J. Cell Biol. 35: 52-53A, November 1967.

3. Hall, W. T. and Feller, W. F.: Biophysical and bioassay studies on milk fractions of genetically similar agent-bearing and agent-free mouse substrains. J. Nat. Cancer Inst. 39: 1155-1185, December 1967.

4. Hall, W. T., Andresen, W. F., and Evans, V. J.: In vitro and in vivo observations on a murine C-type virus. J. Virol. 1: 1238-54, Dec. 1967.

5. Hall, W. T., Hartley, J. W., and Sanford, K. K.: Characteristics of and relationship between C-particles and intracisternal A-particles in cloned cell strains. J. Virol. 2: 238-247, March, 1968.

6. Hall, W. T., Hartley, J. W., Andresen, W. F., Sanford, K. K., and Evans, V. J.: Virus particles and murine leukemia virus complement-fixing antigens in neoplastic and non-neoplastic cell lines. Yearbook of Cancer, 12: (1968) In press.

Serial No. NCI-3226 SAME
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Carbon tetrachloride-induced cirrhosis of the liver in Buffalo and ACI male and female and Fischer female rats of varying ages.

Principal Investigator: Melvin D. Reuber

Man Years:
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives: Study the susceptibility and severity of female and male rats of different ages to cirrhosis of the liver.

Methods: Buffalo female and male rats were 4 day, 1 week, 4 weeks, 12 weeks, 24 weeks, or 76 weeks old at the start of the experiment. They received carbon tetrachloride and corn oil subcutaneously for 12 weeks.

Major Findings: The older male Buffalo rats developed a higher incidence and more severe cirrhosis of the liver than did the younger male rats. With the female rats the 12- and 24-week-old animals had the most severe cirrhosis. In some age groups males developed the most severe cirrhosis; in other age groups the cirrhosis was more advanced in the female animals.

Significance: The incidence of carbon tetrachloride-induced cirrhosis of the liver was higher and the cirrhosis was more advanced in the older animals. The differences related to the sex were dependent upon the age.

Proposed Course: Studies with ACI male and female rats and Fischer female rats have been completed. The data has not been analyzed because the histologic sections have not been received.

Publications:

Reuber, M. D. and Glover, E. L.: Carbon tetrachloride-induced cirrhosis. Effect of age and sex. Arch. Path. 85: 275-279, 1968.

Serial No. NCI-3226-A
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Age and sex and choline deficient cirrhosis and hyperplasia of the liver

Principal Investigator: Melvin D. Reuber

Project Description:

Objectives: Determine the influence of age and sex on the development of cirrhosis and hyperplasia of the liver resulting from a choline deficient diet.

Methods: Buffalo female and male rats 4 weeks, 12 weeks, 24 weeks, or 52 weeks of age were fed a choline deficient, high fat diet for 22 weeks. The degree of cirrhosis of the liver in the various groups was compared.

Major Findings: The slides have been studied for the 12-week-old male and female rats. The cirrhosis is more severe in the males than in the females. There are also more hyperplastic nodules in the males than in the females.

Significance: The results differ from those obtained with a study of carbon tetrachloride induced cirrhosis. There was less difference between the males and females; however, the 24-week-old animals had more severe cirrhosis than did the 12-week-old animals.

Proposed Course: Analyze data for 4 week and 52 week old rats after histoligic sections have been received. See if the hyperplastic nodules are preneoplastic and would develop into carcinomas of the liver.

Serial No. NCI-3228 SAME
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Histogenesis of cholangiofibrosis and well differentiated cholangiocarcinomas in Syrian hamsters given N-2-fluorenylacetamide and N-2-fluorenyldiacetamide, and of undifferentiated carcinomas in Fischer rats given 3'-methyldimethylaminoazobenzene.

Principal Investigator: Melvin D. Reuber

Man Years
Total: 1
Professional: 1

Project Description:

Objective: Study the early lesions of the liver that later develop into carcinomas.

Methods: A. Male and female hamsters were given N-2-fluorenylacetamide or N-2-fluorenyldiacetamide in the diet. They were killed periodically in order to study the development of lesions of the liver.

B. Fischer male rats were given 3'-methyldimethylaminoazobenzene in a low fat, high carbohydrate diet. They were killed at scheduled time intervals in order to study the lesions of the liver.

Major Findings: A. Cholangiofibrosis and cirrhosis preceded the development of well differentiated cholangiocarcinomas in both male and female hamsters. The lesions could be followed from (1) cholangiofibrosis to (2) cholangiofibrosis with atypical cells to (3) small cholangiocarcinomas to (4) large cholangiocarcinomas with metastases to portahepatic lymph nodes.

B. A similar development of lesions was observed in Fischer rats. The carcinomas were undifferentiated or poorly differentiated cholangiocarcinomas.

Significance to Cancer Research: Opie and Firminger have long felt that cholangiofibrosis was a precursor of carcinoma. They did not describe histologic changes taking place in the development of such lesions over a period of time.

Stewart still believes that the experimental animal does not develop cholangiocarcinomas. He refers to such tumors as adenocarcinomas because he feels that they arise from liver parenchymal cells.

Rats given the same carcinogens in the same dosage and diet develop predominantly hepatocellular carcinomas.

It is widely believed that the undifferentiated carcinomas in rats develop from liver parenchymal cells. In this study they developed from cholangiofibrosis.

Proposed Course of Research: Prepare the results observed in the Fischer rats for publication. Study and describe the histogenesis of carcinomas of the liver in rats given 2-7-fluorenylenebisacetamide.

Publication:

Reuber, M. D.: Histogenesis of cholangiofibrosis and well differentiated cholangiocarcinomas in Syrian hamsters given 2(diacetoamino) fluorene and (acetoamino) fluorene. In press. Gann.

Serial No. NCI-3231 SAME
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigation of the role of prolactin in the development of mammary cancer.

Principal Investigator: M. Ben-David

Other Investigators: Collaborative research is carried out with Dr. W. E. Heston, Chief, Laboratory of Biology, and Dr. A. Chrambach, Endocrinology Branch, NCI.

Man Years:
Total: 2-1/2
Professional: 1
Other: 1-1/2

Project Description:

Objectives: Three targets were the objectives of this year's project:
1) To investigate the mechanism of action of perphenazine in its production of abnormal lactation in rats, through simultaneous determination of prolactin in the pituitary and blood, together with histological studies of the mammary glands, ovaries, uterine endometrium, and vaginal smears.

2) To study the influence of the presence of the mammary tumor virus (MTV) on the mammotropic effect of perphenazine in various strains of mice with and without MTV.

3) To try to demonstrate the existence of human prolactin.

Methods: 1) Bioassay of very small amounts of prolactin in the serum and in various bands obtained after polyacrylamide gel electrophoresis. Last year we modified the local pigeon-crop method for prolactin assay into a very sensitive and entirely objective method using H^3-Methyl-thymidine uptake by the crop mucous epithelium only (1). This modification made it possible to assay as low as 1 mU of prolactin and for the first time it was possible to detect prolactin in the rat serum. Moreover, together with Dr. Chrambach we have developed a special technique to detect traces of prolactin in slices of polyacrylamide gel by using a second electrophoresis of one or multiple slices of the same Rf moving into 0.2% agar solution which was subsequently injected over the pigeon-crop for prolactin assay.

2) Histology of mammary gland development (Mammotropic Index, MTI), in mice following treatment with a prolactin releaser. This technique was used especially to study the influence of the mammary tumor virus (MTV) on the

mammotropic effect of perphenazine, an amino derivative of chlorphenathiazine, and a prolactin releaser.

3) Histological study of the mammary glands, ovaries, endometrium, and vaginal smears and RNA determination of the mammary glands after treatment of perphenazine in rats. This method used to elucidate the mechanism of action of the mammotropic effect of perphenazine.

Results: 1) Mechanism of action of perphenazine in rats (partial collaboration with Dr. A. Chrambach). Remarkable and prompt release followed by synthesis and release of prolactin by the pituitary, accompanied by significant increase in the pituitary wet weight and mammary growth and secretion were obtained during 5 days' treatment with perphenazine in Fischer female rats (6). At autopsy on day six, the ovaries were found to be predominantly occupied by corpora lutea. A typical pseudopregnant "lucid zone" appeared in the uterine endometrium and a continuous stage of diestrus ensued which lasted for about 7 days after the end of treatment (2). It is concluded that perphenazine stimulated endogenous secretion of prolactin which in rats acted also as a luteotropic hormone (LTH). Perphenazine was proved to be inactive in hypophysectomized rats (4) which indicates that its action is central and not peripheral. It was also shown that continuous exposure of the animals to the phenothiazine drug must be maintained in order to obtain full mammotropic reaction (3).

2) Mammotropic effect of perphenazine in mice with and without MTV (in collaboration with Dr. W. E. Heston). Various doses of perphenazine following pretreatment with estradiol (estradiol priming) or without such priming, were given to the following inbred mouse strains with and without the mammary tumor virus: RIII (lost RIII agent), RIIIfC3H, DDfB, DD, C3HfB, C3H, C3HfDD, BALB/c, BALB/cfC3H, BALB/cfDD, AfB, A, A (Low), and A (High).

Perphenazine treatment induced higher mammary growth in all animals which carry the MTV, as compared with mice of the same strain but free of the virus. Estradiol priming, however, induced similar mammary growth in both virus-free mice and those carrying the MTV, but genetic differences among the strains of mice in the reaction of their mammary glands to estradiol were clearly shown. It is concluded that the presence of the MTV in mice potentiates the mammotropic effect of perphenazine. Nevertheless, no parallelism was found between the reported susceptibility of various mouse strains to the appearance of spontaneous mammary tumors and their mammary sensitivity to the prolactin releaser, perphenazine (5).

3) Prolactin (in collaboration with Dr. A. Chrambach). In preparation for the human problem, and taking advantage of the availability of lactation in the rat induced by perphenazine, rat prolactin was investigated. The biologically active protein is not visibly stained in electrophoretic patterns of rat pituitaries obtained either at pH^{π}=9.45 (25°C) or 7.8 (0°C); contrary reports in the literature with reference to the pH^{π}=9.45 system have been disproved. However, the pattern of pituitary extracts prepared by adaptation of the serum-procedure of Bates does yield a strong band Rf 0.58 (pH^{π} = 7.8, 10% acrylamide) which is biologically active. The active band of this Rf was demonstrated both in pituitaries of lactating and perphenazine-treated rats.

The pharmacologically induced protein appears thus identical to the naturally occurring one, and the mechanism of action of the drug is shown to directly involve the synthesis of prolactin in the pituitary (see also "Results, part 1"). The identical extract of rat serum yields a vastly heterogeneous pattern with no clearly visible band of Rf 0.58. Only after electrophoresis of multiple gel slices first into a concentration gel of pH^{π} = 7.95 (0°C) and of the stock in 0.2% agar can the serum activity be detected. Its Rf, both in sera derived from lactating and from perphenazine treated animals, is identical to the pituitary hormone.

Human pituitary prolactins of Rf 0.41, 0.48, and 0.53 were demonstrated in a system (pH^{π} = 7.8, 10% acrylamide) in which standard NIH GH exhibits an Rf of 0.36 in its main band. This represents the first direct demonstration of human prolactin different from human growth hormone.

Significance to the Program of the Institute: The role of prolactin in the development of mammary cancer in women is not yet known, especially because it is not known for certain that prolactin does exist in humans and because of lack of a method capable of measuring prolactin in the blood.

We have for the first time demonstrated the existence of human prolactin distinct from human growth hormone. By means of polyacrylamide gel electrophoresis of preparation derived from human pituitary we were able to identify specific bands of a very high prolactin activity with Rfs (0.41, 0.48, and 0.53) distinct from the major band of human growth hormone (0.36). Simultaneous assays of prolactin and growth hormone of these bands are now underway in order to find a band which has high prolactin activity and no or very low growth hormone activity. Isolation of such protein of high prolactin activity is essential for developing radioimmunoassay for human prolactin badly needed in the field of human mammary cancer research.

Proposed Course of the Project: My tenure at NIH will be concluded on June 30, 1968. Afterwards I will return to my former Department of Applied Pharmacology of the Hebrew University of Jerusalem, Israel. I plan to continue to study the effect of prolactin on the mammary growth and carcinoma, especially in RIII and DD mouse strains. Furthermore, I intend to study the correlation between prolactin blood level and mammary cancer incidence among the Jewish Yemenite women who have an incidence of 12.9 and in the entire population which shows mammary cancer incidence of 53.9 per thousand.

Publications:

1. Ben-David, M.: A sensitive bioassay for prolactin using H^3-methyl-thymidine uptake by the mucous crop epithelium. Proc. Soc. Exp. Biol. Med. 125: 705-708, 1967.

2. Ben-David, M.: The role of the ovaries in perphenazine-induced lactation. J. Endocrinol., Article 2471, 1968.

3. Ben-David, M.: Induction of hypothalamic lactation in Fischer inbred rats by depot injection of fluphenazine enanthate. Neuroendocrinology, Article 072, 1968.

4. Ben-David, M.: Induction of lactation in intact and hypophysectomized Sprague-Dawley female rats by perphenazine and its mechanism. Endocrinology, Article 68-3-89.

5. Ben-David, M. and Heston, W. E.: Effect of perphenazine, a prolactin releaser and estradiol of the mammogenesis of various strains of mice with and without the mammary tumor virus. J. Nat. Cancer Inst. (in press).

6. Ben-David, M. and Chrambach, A.: Stimulation of prolactin release and synthesis by perphenazine in Fischer rats. Proc. Soc. Exp. Biol. Med. (in preparation. Abstract will be presented at the Third International Congress of Endocrinology, Mexico City, 1968, Article 187.)

Serial No. NCI-3235 SAME
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Nephrosis and hepatic lesions in rats given calcium disodium edetate

Principal Investigator: Melvin D. Reuber

Man Years
Total: 2
Professional: 1
Other: 1

Project Description:

Objectives: Evaluate the factors affecting the renal toxicity of calcium edetate.

Methods: Animals were given daily intraperitoneal injections of calcium edetate for 21 days. The following factors were studied:

A. Strain-random bred rats (Osborne Mendel, Wistar, Sprague-Dawley, Black Rat, 3 months of age)

B. Age in male and female rats (4 day, 4-, 12-, 24-, and 52-week-old rats)

Major Findings:

A. There were minimal lesions of the kidneys in Wistar and Black Rats, and moderate changes in Sprague-Dawley and Osborne Mendel rats. Severe lesions developing in inbred rats were not observed.

B. Twenty-four- and 52-week-old and 4-day old male rats died much sooner with severe nephrosis. Four and 12-week-old animals had minimal renal lesions. The female animals were the same, except for the 24-week-old rats that had minimal lesions.

An incidental finding was hydropic change and chronic inflammation in the livers of 4-week-old males and females.

Significance: Calcium disodium edetate is used for the treatment of lead poisoning. Four cases of death from nephrosis have been reported.

Proposed Course: The 24-week-old male rats were more susceptible than females of the same age. Studies will be done to see if this difference is related to sex hormones.

Proposed Course (continued)

The effect of an acute infection on the development of the hepatic lesion will be studied.

Publications:

1. Reuber, M. D.: Severe nephrosis in older male rats given calcium disodium edetate. Arch. Environmental Health 15: 141-146, 1967.

2. Reuber, M. D.: Hepatic lesions in young rats given calcium disodium edetate. Toxicol. & Applied Pharmacol. 11: 321-326, 1967 (Sept.).

Serial No. NCI-3236
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Morphologic and biologic correlation of hyperplastic and neoplastic lesions in hybrid and inbred mice with the yellow gene

Principal Investigator: Melvin D. Reuber

Other Investigators: W. E. Heston

Project Description:

Objectives: Correlate the morphology of hyperplastic and neoplastic lesions with their biologic behavior.

Methods: Perform laparotomy and biopsy hepatic lesions for autologous and isologous transplants at 3, 6, 9, 12, and 14 months. Carry out transplants when animals are killed or die.

Results: Hyperplastic and lesions between 3 and 7 mo. have been transplanted isologously and autologously.

Eight carcinomas believed to be unusual have been found histologically and reported in the literature. They are felt to be poorly differentiated cholangiocarcinomas. Previously such carcinomas have not been described as occurring in mice either spontaneously or following administration of carcinogenic chemicals.

Further detailed study has revealed approximately 30 more such carcinomas.

Proposed Course: Continue to observe transplanted hyperplastic lesions and carcinomas. Transplant into second and later generations.

Study the ultrastructure of these lesions by electron microscopy.

Publications:

Reuber, M. D.: Poorly differentiated cholangiocarcinomas occurring "spontaneously" in C3H and C3HxY hybrid mice. J Nat Cancer Inst 38: 901-907, June 1967.

Serial No. NCI-3238 (NEW)
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Growth Hormone and Hepatic Carcinogenesis in Rats Given N-2-fluorenyldiacetamide

Principal Investigator: Melvin D. Reuber

Project Description:

Objectives: Extend previous studies on the role of hormones in hepatic carcinogenesis to include the effect of growth hormone.

Methods: Adult intact and castrated male and female rats received 10.025% N-2-fluorenyldiacetamide in the diet along with subcutaneous injections of growth hormone. The hyperplastic and neoplastic lesions of the liver were evaluated.

Major Findings: Growth hormone increased the incidence of carcinomas and cirrhosis in both castrated male and female rats, but it did not exert any effect in the intact animals.

Significance: These results are similar to those observed previously with progesterone. Both growth hormone and progesterone are weakly anabolic hormones. The results are not striking as with norethandrolone or testosterone - strong anabolic compounds.

Proposed Course: Study role of prolactin in hepatic carcinogenesis in both castrated and intact males given carcinogen.

Serial No. NCI-3239 (NEW)
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Age and Sex and Hepatic Carcinogenesis in Rats Given Diethylnitrosamine (DEN)

Principal Investigator: Melvin D. Reuber

Project Description:

Objectives: Study role of age and sex on hyperplastic and neoplastic lesions of the liver produced by DEN.

Methods: Buffalo female and male rats were 4 weeks, 12 weeks, 24 weeks, or 52 weeks old at the start of the experiment. They received 0.0114% DEN in the diet for 26 weeks. Surviving animals were killed at the end of 32 weeks.

Major Findings: The younger animals were strikingly more susceptible to the development of carcinomas of the liver than were the older animals. They did not survive as long, they had more carcinomas per liver, larger carcinomas, and more metastases to the lungs. Sarcomas were also observed in the younger animals.

The differences in males and females of the different ages were not significant.

Incidentally, carcinomas of the esophagus were also seen more frequently in younger animals of both sexes.

Significance: Males are more susceptible to the development of hepatic carcinomas induced by other hepatic carcinogens. The other carcinogens are soluble in lipid, whereas diethylnitrosamine is water-soluble.

The greater susceptibility of young animals has been noted with N-2-fluorenyldiacetamide.

Proposed Course: Repeat the experiment with 6 month old animals. Since they develop a small number of hepatic carcinomas, they may serve as a model for the study of carcinomas of the esophagus.

Serial No. NCI-3245 (NEW)
1. Laboratory of Biology
2. General Biology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on the transplantation of lesions in hepatic carcinogenesis in rats

Principal Investigator: Melvin D. Reuber

Other Investigators: S. Odashima

Project Description:

Objectives: Transplant hyperplastic lesions intrasplenically, subcutaneously, and intramuscularly into autologous and isologous hosts and intraperitoneally and intrahepatically into isologous hosts. Determine whether such lesions will grow in these sites, and if they do will they retain their histologic pattern or progress on to neoplasia.

Methods: Buffalo and Wistar male rats were fed 0.025% N-2-fluorenyldiacetamide in the diet. They were killed after 8, 12, 14, 20, 23, and 26 weeks and transplants were done as mentioned previously.

Major Findings: Hyperplastic cells sometimes survived when transplanted intrahepatically and developed degenerative changes, but did not grow on transplantation to any site. In isologous hosts, small well-differentiated carcinomas grew only after intrahepatic transplantation. They grew better in male than in female animals.

Significance: Hyperplastic cells and small carcinomas will progress to a more advanced stage, i.e., large carcinomas only in the liver of the animal given the carcinogen. By contrast, the large carcinomas are easily transplanted.

Proposed Research: Study the ultrastructure of the hyperplastic lesions, as well as the carcinomas, with the electron microscope. Perhaps study the role of immunologic factors in the failure of the hyperplastic lesions to grow on transplantation.

Publications:

Reuber, M. D. and Odashima, S.: Further studies on the transplantation of lesions in hepatic carcinogenesis in rats given 2-(diacetoamido)fluorene. Gann 58: 513-520, 1967.

NCI Serial No. 3211 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies, by tissue culture and cell culture methods and by amplifying methods, of the malignant cell and of the normal cell from which it arises; studies on the mechanism of transformation of the normal cell into the malignant cancer cell; studies on associated significant subjects.

Previous Serial Number: Same.

Principal Investigators: V. J. Evans, K. K. Sanford, B. B. Westfall, J. C. Bryant, R. Cornell, J. T. Mitchell, R. R. Gantt, and R. Parshad.

Other Investigators: See individual project reports Nos. 3211 to 3215, 3220, 3233, 3234, and 3237.

Man Years: July 1, 1967 through June 30, 1968
Total: 22-3/4
Professional: 11
Other: 11-3/4

Outline of Activities: The long-term objectives of the personnel of the Section are described in the general title above and in detail in the individual projects. To carry out the objectives considerable time has been spent in past years, and will continue to be spent as required, to maintain and to develop superior technology and instrumentation, to advance all current problems and to anticipate maintaining eminence in the primary interests.

Some of the inordinately time consuming but necessary details on acquiring and evaluating physical equipment required for long-term, large scale antibiotic free tissue culture work are mentioned here because they result in recently acquired information that other investigators in related fields might find useful.

Considerable rebuilding and rearrangement has been done within the suite to incorporate modern equipment needed in tissue culture. An example of this is the current installation, under a research and development contract, of a medium capacity mechanical washer and a dryer for more efficiently cleaning and drying an unusually wide variety of specialized laboratory glassware for tissue culture. The instrument has been evaluated for approximately one year. The quality of washed glassware has been excellent.

In fact, overall, the product is superior to hand washed glassware. The bulk of glassware it turns out is one of the reasons making it possible for us to carry out certain biochemical studies requiring large numbers of culture vessels and ancillary glassware. To date every piece of glassware constantly used in tissue culture work can be accommodated because of the design of racks developed by us to hold the glassware. The piece of equipment is so satisfactory we can recommend it safely to investigators over the country who have made inquiry regarding it and who await our evaluation. It is a much more flexible instrument than any other laboratory washer known to us. Further, it was fabricated from 316 stainless steel. Plans are made to incorporate this washer in the new area we will occupy in Building 37 despite the fact that the square foot area required by it and the dryer and racks is practically an entire small module.

Considerable time has had to be devoted to designing an integrated suite for coordinated activities in the new laboratory for Building 37. This has required exploration of ways and means of obtaining functioning and miniaturized equipment for limited space for long-term, large-scale tissue culture work carried on without antibiotics. Investigation of (1) appropriate autoclaves, wet and dry, safety hoods for hazardous work to cells and operators, and (2) high efficiency accurately temperature controlled incubator space are of high priority. (3) Our present three sterile room areas have required substitution by laminar flow, air containment boxes or safety hoods and benches particularly designed to conserve space and to avoid hazard to both operator and long-term cultures carried without antibiotic. Exploration of such units commercially available has proven none to be satisfactory, but by Dr. Bryant's and Dr. Evans' working with the biohazard engineer, Mr. W. E. Barkley, and with Mr. Terence Martin, a design is being worked out. Our requirements for such containment boxes and benches, because we do not use antibiotics in cultures, put greater demands on the ultimate efficiency of such a box than is required by less precisely controlled work. (4) Our three large high-silica iron kettles for hot sulfuric and nitric acid have proven highly efficient for precleaning critical glassware; we know of no other way that insures complete removal of all protein and carcinogen residues from glassware. The facts that the special acid-resistant kettles are about 15% silica and hence are relatively fragile, and that the fume hood must be highly efficient may present serious problems in setting up similar new acid kettles in our suite in Building 37. The company that originally furnished our present kettles appears to be the only source of such kettles, and then only on a custom basis in order to meet the strict requirements of highly specialized tissue culture glassware.

One new device is being tested for growing larger amounts of tissue cultured cells without antibiotics and as surface substrate cultures. Another device, automated for fluid change in order to save manpower, has been located. This laboratory has been the first asked to test this particular instrument in actual culture use.

A new microphotography unit to replace the over 25 year old unit has been custom designed in consultation with us and has been purchased. The unit is designed to use less space, to be able to use smaller less expensive

film, and to have optics both bright field and phase satisfactory for photography through the specially designed flasks used in this laboratory. The research division of one outstanding optics company has custom designed two new phase plate substitutes for long working distance condensors that are of paramount value to tissue culture work and has asked us to evaluate them for photography under our actual unique culture conditions. This came about after their senior research optics engineer made a site visit to our laboratory prior to their beginning to redesign their microcinephotography units.

The laboratory has also been involved in attempting to design specifications of a simple microcinematograph and to test many models and special optics for use with cells grown in special design flasks. The instrument is specially planned to be operated by biologists rather than a professional photographer and it conforms to the fact that only limited space is available for placement of the instrument in Building 37.

Project activities are listed under serial projects 3211, 3212, 3213, 3214, 3215, 3220, 3234, and 3237.

SAME
Serial No. NCI 3212 - Project 1
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project 1, Title: Studies of cell nutrition in vitro

a. Cultivation of freshly explanted C3H mouse embryonic fibroblasts on chemically defined medium NCTC 135

b. Studies on the nutritional requirements of a neoplastic C3H mouse embryo cell strain for pyrimidine and purine nucleosides and other related compounds

c. Adaptation of a non-neoplastic cell line, NCTC 4823 growing in medium NCTC 135 supplemented with serum

d. Assay on stored media, sources of distilled water and commercially available chemically defined media

e. Studies on the nutrition of L-1210 mouse leukemic cells

Principal investigators: V. J. Evans and J. C. Bryant

Other investigators: F. M. Price, H. A. Kerr

Man years: See NCI serial No. 3211

Cooperating units: Nucleic Acid Section, Laboratory of Biochemistry, NCI
Dr. J. Rotherham and Dr. W. E. Schneider

The American Type Culture Collection, Cell Repository Laboratory

Project Description (a): Cultivation of freshly explanted C3H mouse embryonic fibroblasts on chemically defined medium NCTC 135.

Objectives: To continue study to improve methods to prolong survival and growth of primary tissue so that adequate biological material can be produced over an indefinite period of time. The tissue is being used to study the effect of medium composition, type of culture, rate of growth, chromosomal changes, biochemical changes, subculture on the culture transformation of normal cells to tumor producing cells in medium NCTC 135. The ultimate goal is to study and control neoplastic transformation under rigid culture conditions.

Methods Employed: C3H mouse embryos (11-13 day) were used as the tissue source because: 1) they provide the easiest means to obtain a uniform pool of tissue suitable for comparison of results over a long period of time; 2) they are assumed to have the greatest potential for growth in tissue culture, and 3) they offer all cell types for possible selection for growth in a completely chemically defined medium. The embryos were minced and used to inoculate T-flasks. Tissue was removed frequently from the T-flask.

Major Findings: Five cell lines (Exps. 19, 26, 28, 32, and 33) have been under investigation for a period of up to 4 years. All have proliferated sufficiently to allow repeated removal of tissue for animal injections and inoculation of T-15 daughter cultures, although 2 of these lines (Exp. 26 and 28) could never be subcultured successfully. For all these cell lines the initial population was largely rather small, transparent fibroblasts. After approximately 200 days in vitro, a small epithelial-like cell appeared in scattered patches. These epithelial-like cells grew under the original layer of fibroblasts which then sloughed off the glass substrate into the medium. After sloughing, cells of the original fibroblast-like layer were no longer viable. Our observations on the behavior of these two cell types has been verified by time-lapse microcinematography. In flasks (Exp. 26 and 28) that were left undisturbed, the small epithelial-like cells eventually became the only surviving cell type. These cells proved to be very fragile and were never successfully removed from the original parent flasks. In cultures in which tissue removal was frequent these apparently fragile epithelial-like cells composed only a small percentage of the total cell population. In one like (Exp. 19) after 17 months of frequent tissue removal, a larger, less transparent fibroblast-like cell completely dominated the cultures and became the only apparent cell type. These larger, more granular fibroblast-like cells have a much faster growth rate and can be subcultured weekly. They have also been tested for their ability to grow in less complex NCTC media and show a requirement for both inosital and vitamin B12. A second line (Exp. 33) has become established after 26 months in vitro. The predominant cells in these cultures are relatively small bi-polar cells, much like the original population. Cells in experiment 32 now appear to be becoming a rapidly growing cell line although the cultures cannot always be subcultured at 7 day intervals.

Three of the cell lines (Exp. 19, 26, and 33) have produced tumors after 411, 169, and 902 days respectively. Although tumor production coincides with time of rapid proliferation for the cells in Experiment 19, there does not seem to be a similar correlation for the cells in Experiments 26 and 33. Although cells of Experiments 26 and 33 produced tumors after 169 and 902 days in vitro they did not show an increase in cell growth at that time. Cells of Experiment 32 have still not produced tumors after 1367 days in vitro, but are still being tested. Cells of Experiments 19 and 33 have both been shown to contain a murine leukemia virus. Experiment 32 is presently being tested for presence of virus.

Significance: These findings support the concept held in this laboratory that chemically defined medium of the NCTC series is adequate for the continued growth of freshly explanted cultures. The ability to grow long-term cultures of primary tissue in chemically defined medium is enhancing the

chances of obtaining results from experiments investigating the malignant transformation and the nutritional requirements of a wide spectrum of fresh tissue. The appearance and disappearance of the "new" epithelial cells in the static cultures and the ability to subculture the less transparent faster growing fibroblast-like cells may hold special significance in the malignant transformation.

Virus particles from Experiment 19 have been isolated and a study made to determine whether this leukemia virus may play a role in converting normal cells to neoplastic cells. For further information on this matter see serial No. 3212, project 3.

Proposed Course: Cultivation of primary tissue in chemically defined media will be continued and data on morphology, nutrition, metabolism, growth rate, and chromosomal differences will be collected and summarized for correlation, if possible, with the malignant transformation. Investigations to gain more rigid control over the cultures will be carried out with the expectation of understanding, and thus controlling the malignant transformation. Efforts will be made to obtain several clonal populations from these mixed populations to see if there is any special significance in neoplastic transformation of selection of cell types.

Project Description (b): Studies on the nutritional requirements of a neoplastic C3H mouse embryo cell strain for pyrimidine and purine nucleosides and other related compounds.

Objective: To determine which purine or pyrimidine nucleoside and/or other related compounds are specifically required nutritionally for a neoplastic mouse embryo cell strain. This project will provide background information for a simultaneous biochemical project to determine the role of these nucleosides in the metabolism of these cells in vitro. For this biochemical information see the project of Dr. Jean Rotherham, NCI Serial No. 397, and Serial No. 3212, project 3.

Methods Employed: These cells had previously been adapted to growth in medium NCTC 117 which is like medium NCTC 135 but lacks 6 coenzymes, 3 nucleic acid derivatives and sodium acetate. Medium NCTC 137 was devised to test nutritional requirements of the nucleosides and other related compounds. Medium NCTC 137 is like medium 117 but has no nucleic acid derivatives and no vitamin B12. Vitamin B12 was omitted from medium 137 because previous information indicated that it might be involved in nucleic acid synthesis and because we had found previously that this particular cell strain required vitamin B12 in the absence of deoxyadenosine and deoxyguanosine.

Stock solutions of 8 nucleic acid derivatives, orotic acid, vitamin B12, and folic acid were prepared and test media involving various combinations of these components in medium NCTC 137 were prepared and placed in replicate T-15 cultures of these cells to determine the combinations which would support prolonged growth. All experiments were repeated.

Major Findings: The initial tests were made with various combinations of the pyrimidine nucleosides and vitamin B12. No culture in such test media without vitamin B12 lived for more than 5 culture generations. However, the addition of vitamin B12 alone to medium 137 was not adequate to support prolonged growth past 4 culture generations. Three pyrimidine nucleosides, when used in combination with vitamin B12, did support growth of these cells as continuously growing long-term cultures. These were thymidine, deoxycytidine, and 5-methyl deoxycytidine. With vitamin B12 present cytidine, uridine, deoxyuridine, or orotic acid did not support growth but were not toxic at 0.04 mM/l. These findings suggested that these cells could convert deoxycytidine, but not cytidine or uridine, to thymidylate.

Further tests have been made with the pruine nucleosides, deoxyadenosine, and deoxyguanosine, and with folic acid. It was found that the combination of deoxyadenosine, deoxyguanosine, and vitamin B12 would not support prolonged growth of these cells, although when either or both of these purine nucleosides were used in combination with thymdine or deoxycytidine, prolonged growth was obtained. Studies by other investigators have shown that folic acid, in higher concentration than we usually use in our media, has been found necessary in some systems. We, therefore, tried folic acid in a 10x concentration and found that in combination with vitamin B12 a 10x concentration of folic acid would replace all pyrimidine nucleosides. And while the combination of deoxyodenosine and either cytidine, uridine, or deoxyuridine would not support prolonged growth of these cells, the addition of 10x concentration of folic acid alone, or the combination of these two components was not sufficient to support prolonged growth of these cells. The combination of 10x folic acid with adenine, adenosine, or deoxyribose also did not support growth of these cells.

Significance: The results have not only indicated which nucleic acid derivatives are necessary for growth of this malignant cell strain, but may also be an indication of some of the metabolic reactions that these cells are able to undergo. These results, when coupled with the results of biochemical studies to determine the metabolism and utilization of the nucleic acid derivatives and related components, provide information about the growth of malignant tissue in comparison with that of paired nonmalignant tissue. The hope would be to explore under controlled conditions any changes in biosynthetic capacities from normal to neoplastic cells during culture, in order to understand and control the "neoplastic transformation phenomenon".

Proposed Course of Study: Attention will be turned now to nutritional tests involving a non-neoplastic cell strain for comparison purposes with the neoplastic cell line. Previously comparable studies could not be carried out since the non-neoplastic cell line was not adapted to growth in chemically defined medium NCTC 135 unsupplemented with serum. The adaptation of the non-neoplastic cell line to growth in medium NCTC 135 is discussed in serial 3212, project 1, c.

Project Description (c): Adaptation of a non-neoplastic cell line, NCTC 4823 growing in medium NCTC 135 supplemented with 10% (v/v) FCS to growth in medium 135 unsupplemented with serum.

Objectives: To adapt a non-neoplastic cell line growing in medium NCTC 135 and 10% FCS to growth in medium 135 without serum.

Methods Employed: NCTC 4823 cultures which had been grown in NCTC 135 supplemented with 10% FCS for over 2 years were adapted to growth in NCTC 135 by gradually reducing the serum content of the culture fluid.

Major Findings: As has been the case with most of our cell lines which have been adapted to growth in chemically defined medium NCTC 135 after initiation and growth in serum containing medium, a considerable length of time was necessary before the cells became fully adapted to growth in unsupplemented NCTC 135. Thus far 4 substrains, NCTC 5981, 6000, 6011, and 6196, have been adapted to growth in medium NCTC 135 and a fourth, NCTC 5981, to growth in medium NCTC 135 with an additional 10x concentration of folic acid. Two of these substrains, NCTC 6000 and 5981, required a reduction in serum content down to 1/8% over a period of 380 days before they could be cultivated in chemically defined medium alone. Chromosome analyses have been made in both these substrains and in general they differ from chromosomes of the original cells grown in serum-containing medium. For further chromosome details see Serial No. 3233. NCTC 6000 has been examined by electron microscopy for cellular detail and presence of viral C particles (See Serial No. 3237). These cells have also been injected into isologous animals as a test for neoplastic transformation but insufficient time has elapsed to determine whether these cells are neoplastic or not. Substrains NCTC 6071 and 6196 required a longer adaptive period than those of 6000 and 5981 before they could be grown in completely chemically defined medium. The total time was 421 days and it involved a further reduction in concentration of serum content to 1/12% before final adaptation could be achieved. Chromosome analysis of these substrains has shown them to be different from each other and from NCTC 5981 and 6000.

Significance: We have shown by these studies that a non-neoplastic cell line can be adapted by slow serum depletion to growth in chemically defined medium and that material could be produced for tests for neoplastic transformation. Chromosome analyses of the adapted lines has shown that all the adapted lines differ in some degree from the parent and in some cases from each other, but are not as dramatically altered as when the same cells were exposed to the chemical carcinogen 7,12 dimethybenzanthracene. This leads us to believe that some selection process may have taken place during the adaptation. The difference in chromosome patterns in the adapted lines raises the question whether removal of fetal calf serum is releasing the cells from a possible repressor-like action in the serum. This repressor-like action, if proven, could be responsible for the fact that the cells removed from it show altered chromosome patterns when removed from fetal calf serum. This could possibly account for the differences between chromosome patterns of the adapted lines since two of the lines were completely removed from serum more than one month earlier than the other two.

Proposed Course of Study: Injections of all sublines will be made periodically to determine if any or all sublines are now neoplastic or have or will become neoplastic in the absence of the fetal calf serum. We are presently

carrying subline NCTC 6000 in medium NCTC 135 with 10% gelding horse serum in order to see whether we can rapidly convert it neoplastically. The adapted cell lines will also be used in studies involving chemical and or viral carcinogenesis in the event that neoplastic conversion does not occur "spontaneously", i.e., with horse serum. Results of the tests for neoplasia will be correlated with chromosome analyses if correlations can be made. These adapted cells, if they remain non-neoplastic, will also be useful in studies comparing neoplastic cells grown in chemically defined media with non-neoplastic cells in the same media, especially in studies involving nucleic acid metabolism done in collaboration with Dr. Jean Rotherham, Serial No. 3212, projects 1 and 3.

Project Description (d): Studies with strain 2071-L mouse fibroblast cells, described in last year's report, were continued on 1) the effect on cell growth of aging during storage (4°C) of our chemically defined medium NCTC 135, and 2) the effect on cell growth of distilled water from different stills in different laboratories when used for preparation of medium NCTC 135. Studies with 2071-L cells, also reported last year, comparing certain commercially available NCTC media with our own preparations of these media, have been completed.

Objectives: To study further 1) whether prolonged storage of chemically defined medium at 4°C alters the effect of this medium on cell growth; 2) whether distilled water from commercially available stills can be used to prepare a culture medium that gives satisfactory growth; and 3) whether the growth of several established strains of cells in commercially available NCTC media is equal to that in our own preparations of NCTC media.

Methods Employed: Our replicate culture method with T-15 culture during 7-day experiments or a modification of it, with strain 2071-L neoplastic mouse cells, were used for the three subprojects. Chemically defined medium NCTC 135 was used for subprojects 1 and 2, while several other NCTC media were also tested in subproject 3. The experiments were refined by having four replicate cultures per treatment instead of the two used in experiments reported last year. The Coulter Counter method was used for all three subprojects during this year, since with strain 2071-L cells the cell counts were found to be practically the same as those with the nuclei count method that we have used, and the electronic counts were more easily carried out. Other details on methods were included in last year's report.

Major Findings:

1) Within the storage age limit tested (18 months) during the past year, medium NCTC 135 (sterile, kept at 4°C) retained its growth potential undiminished. Such differences as did show up were not associated with storage age. This conclusion generally confirms last year's results, where 2 years seemed to be the storage age limit.

2) Source of distilled water was associated with significant differences in cell population at 7 days. Lots of medium 135 prepared with 3x-distilled water from our present long-used still had somewhat higher

growth potential than did medium 135 prepared with 1x to 3x-distilled water from other stills in other laboratories; deionized water was used for one of the groups tested and ranked low.

3) A paper is being published (May 1968) on some of our experiments comparing commercially available preparation of our NCTC media with our preparation. This paper reports that while satisfactory growth of 3 cell strains was obtained with the commercial powder-media preparations at high inoculum level, the growth rates at low inoculum levels were lower than in medium prepared and routinely used in our laboratory. The growth potential was also reduced with prolonged culture in the powder-media preparation. The additional work described in last year's annual report, where cell growth in the commercially available preparations was significantly less than that in our own preparations, confirms that now being published.

Significance: The experiments on storage aging of chemically defined medium NCTC 135 reported here, confirming previous work in this laboratory, emphasizesthat the growth potential differences between different lots of the same medium can arise from factors other than storage. In practice, however, we routinely use chemically defined medium that has been stored less than 2 to 3 months, partly because we prepare it and use it in such large volumes.

The often neglected importance of the highest quality of distilled water for chemically defined media was pointed up by our experiments reported last year where a large capacity Corning still in our laboratory produced water from which the media had a comparatively low growth potential. This year the distilled water from several other sources was also slightly inferior to that we use in this respect. As a result of these experiments our laboratory is planning to take our present long-used still with us (see Serial No. NCI-3212) into Building 37.

Commercially available chemically defined media, at least the powdered forms that we have tested, appear to fill a need for the many tissue culture laboratories not in a position to prepare their own media, particularly when serum is used as a supplement. Where no serum or hydrolysate supplement is used, and where the laboratory has high quality distilled water available, commercially available powder-media might be adequate even though in our hands our own preparations have been consistently superior in terms of cell growth.

Proposed Course of Study: Our results on the aging of media in storage will be written up as a short note. One aspect that possibly merits some further study would be to check our results with another established cell strain.

The AG-10 Corning still may be checked further, depending on feasibility of running the distillate from it through the still again, to produce 3x-distilled water, or to put in another still in tandem. The Corning AG-10 still's comparatively large capacity would justify some further testing.

Our results with commercially available chemically defined medium preparations do not justify further work for any advantage to us in our laboratory in the near future. Such tests are extremely time consuming, but have been

an endeavor to obtain security in depth for sources of media other than those prepared by ourselves.

Project Description (e): Studies on the nutrition of L-1210 mouse leukemia cells.

Objective: To isolate and grow in tissue culture various lines of L-1210 mouse leukemia cells. These lines, all of ascites origin, are being cultured in chemically defined media, some supplemented and some unsupplemented with serum.

Methods Employed: Cells were removed from mouse ascites tumors and planted in roller tubes. Initially the culture medium was NCTC 135 plus 10% (v/v) gelding horse serum. Cultures are being adapted to growth in chemically defined medium NCTC 135 by gradually reducing the serum content of the medium. Following adaptation to growth in NCTC 135, studies using less complex NCTC media are to be carried out to define further the nutritional requirements of these cells. During all nutritional studies the cells are subjected to various biochemical assays, NCI Serial No. 3214.

Major Findings: To date the only L-1210 cultures to grow continuously are those supplemented with either large or small amounts of serum. The work of other investigators has indicated the need of these cells for high concentrations of folic acid. Our medium NCTC 135 contains 0.025 mg/L of folic acid. When 4 times that amount was added to NCTC 135 these cells still would not continue to proliferate past 2 or 3 subcultures. At the present time several cultures are growing in medium NCTC 135 supplemented with a 10x concentration of folic acid. Thus far these cultures have been maintained through 3 transfer generations with good growth and as yet show no signs of depletion. It is still possible, however, that these cultures may eventually die out. Biochemical assays have shown that these cells deplete the glucose from the medium quite rapidly and it may be necessary to add additional glucose to NCTC 135 in order to grow these cells without a serum supplement.

Significance: These cells have proved to be useful for biochemical studies and as test cells of the ascites type for chemotherapeutic agents for cancer treatment. Some of these cells are presently being used in our laboratory to test the theory proposed by other investigators that there is a correlation of the activity of an agglutinin in a wheat germ lipase extract with neoplastic and non-neoplastic cells (see Serial No. 3234).

Proposed Course of Study: To continue studies in progress and to study the nutritional and biochemical behavior in chemically defined medium. Chromosomal characteristics will be studied and efforts will be made to determine if *in vivo* differentiating markers still persist *in vitro*. In our laboratory the L-1210 cells will be used for continuing studies on mass cultures in fluid suspension continuously fed systems (serial 3220). They are particularly useful because they are so lightweight and if methods can be developed to hold them back in the culture vessel any cell ought to be able to be retained without spilling over into the used medium vessel.

NCI Serial No. 3212, Project 2 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project 2, Title: Cell transformations in vitro.

a. Studies on freshly explanted C3H mouse embryo cells cultured on chemically defined medium.

b. Comparative studies on the spontaneous malignant transformations in vitro of freshly explanted C3H mouse embryo cells in chemically defined medium NCTC 135 supplemented with either horse serum of fetal calf serum.

c. Studies on the spontaneous neoplastic transformation of a non-neoplastic long-term cell line cultivated in medium NCTC 135 supplemented with 10% v/v fetal calf serum.

d. Studies comparing the effect of adult and fetal serum on the neoplastic transformation in vitro.

e. Studies on the effect of various horse and fetal calf serum fractions on the neoplastic transformation in vitro.

f. Studies on the transformation with a chemical carcinogen 7,12-dimethylbenzanthracene of strain NCTC 4823, a non-neoplastic line from minced C3H mouse embryos.

Principal Investigators: V. J. Evans, W. F. Andresen, F. M. Price, and J. T. Mitchell.

Other Investigators: J. L. Jackson, R. H. Altland, and J. R. Martin.

Cooperating Units: Dr. T. B. Dunn, Laboratory of Pathology, National Cancer Institute.

Man Years: See NCI-3211

Project Description (a): Studies on spontaneous malignant transformation of freshly explanted C3H mouse embryo cultured in chemically defined medium NCTC 135.

Objectives: 1) To investigate whether or not C3H mouse fibroblasts grown in chemically defined medium NCTC 135 with no added serum or any supplement will produce tumors upon injection into the strain of origin as do those grown in

serum supplemented media, and if so, how long a period of cultivation in vitro is required before the cells show the capacity to produce tumors.

2) To progress toward the total control of culture conditions, by controlling culture technology and composition of the culture medium in order to investigate under more closely controlled conditions the long recognized phenomenon of malignant transformation of cells cultured in vitro and to use for neoplastic conversion studies to avoid complications of serum.

3) To see if chromosomal changes in these cultures, if any, are similar to those in cultures grown in serum supplemented media and to correlate such changes if possible to the malignant transformation phenomenon.

Methods Employed: Thirteen day old C3H/HeN mouse embryos were minced and established and carried as described in JNCI (Feb. 1967 pp. 169-183). Because these cells in chemically defined medium have proven sensitive to subculture by mechanical means, they were maintained in the original isolation T-30 flask and tissue was periodically removed for either intraocular implants or transfer to T-15 flasks. In the last two experiments (of a total of five) large numbers of isolation flasks were set up so that flasks could be periodically sacrificed for chromosomal analysis by a technique described earlier, J Nat Cancer Inst 38: 169-183, 1967, and 32: 681-707, 1964.

Major Findings: Five cell studies (Exps. 19, 26, 29, 32, and 33) have been observed for many months. Two strains (Exp. 19 Series 4705, and Exp. 33 series 5047) became rapidly growing established lines. A third (Exp. 32 series 5009) appears to be becoming an established line. One of the five (Exp. 19) is described in J Nat Cancer Inst 38: 169-183, 1967. Three distinct cell types appeared in the course of final development of mouse embryo strains into established cell lines in chemically defined medium. In the early months of culture in vitro, in strains of cells of mixed cell types, the mixture is gradually replaced by a fibroblast-like cell type(s) which becomes finally the only morphologically apparent cell type in culture. After approximately 200 days in culture small colonies of small epithelial-like cells appear in the culture and, although these cells are extremely fragile and cannot be effectively transferred, they will, unless destroyed, undermine and destroy the original fibroblast population. If these epithelial cells are periodically destroyed, a third cell type (usually fibroblastic) which has a rapid growth rate and more granular cytoplasm, appears after approximately 400 days in vitro and rapidly becomes the dominant cell type in culture. While the appearance of the first two cell types was uniform and constant in all five strains, the third appeared only in cultures in which the epithelial cells were destroyed and thus prevented from undermining the initial fibroblast population (Expt. 19, 32 and 33). The attempts to correlate the appearance of cell types, chromosome complement, and age in vitro with tumor production was difficult because the age in vitro at which tumors were first produced and the incidence of production were very inconsistent. Exp. 19 cells produced tumors after 411 days in vitro, when the rapidly growing fibroblast-like cell type was rapidly becoming the dominant cell type in culture as the result of destruction of the small epithelial-like cells. Exp. 26 cells produced a single transplantable tumor after 169 days in vitro when only the initial fibroblast cell type(s)

was apparent; but the cells of this experimental series failed to become an established line even after 25 months in vitro, when the strain was lost. Exp. 28 cells failed to produce tumors and did not become established; the strain was lost after 21 months in vitro. Exp. 33 cells finally produced tumors from cells 900 days in vitro, and as pointed out, carries the murine leukemia virus; it has become a rapidly growing established cell line after 27 months in vitro. Exp. 32 cells have failed to produce tumors after 31 months in vitro but now appear to be becoming an established cell line. It, too, will be continuously tested. Also, a virus, which has tentatively been identified as a murine leukemia, has been found in two of the lines (Exp. 19 and 33) which have become established lines, and the third line (Exp. 32) is now under multiple testing.

Certain incidental observations were made during this study. The small epithelial-like cell contains keratin and is thought responsible for the appearance of stratified squamous epithelium (basement membrane and stratified layers) in the intraocular implants that were not neoplastic. Evidence indicates that these cells are epithelial cells capable of producing a complete skin structure in vitro and in vivo. Thus the cells have retained differentiated function in vitro and for many months which is most unusual. Minced embryo tissues which were never cultured in vitro but directly implanted into the anterior eye chamber produced perfectly normal highly differentiated structures, i.e., hair follicles and bone with marrow, after one year in the host animal.

The two later experiments (32 and 33) after approximately 32 and 31 months growth in T-30 flasks have, in addition to periodic intraocular implants, had periodic chromosome analysis. These analyses indicate that after 276 days (Exp. 33) and 312 days in vitro (Exp. 32) these cultures were still diploid (having 90% or more of the cells in the diploid range). These cultures have an obvious increased stability of chromosome numbers as compared to those grown in sera supplemented media. However, at 409 days or 133 days later only 75% of the cells in Exp. 33 in a very small sample were still diploid by count. Additional determinations have not been made and it cannot be said that the cell line underwent for certainty a chromosome change from diploidy. One of these lines, 5009, judged by EM to contain leukemia particles but not yet proved by CF tests, is to be injected at which time a new analysis of chromosomes will be correlated with these injections to compare with those of 4705. In Exp. 19, cell growth became more rapid after 14 months in culture and could successfully be used to inoculate daughter cultures which were routinely subcultured at 14 days and later at 7 day intervals. Chromosome examination of two daughter cultures revealed a high degree of aneuploidy with a hypotetraploid mode and with only 7% and 6% of the cells in the diploid range. The total age of the cultures giving this information was 448 and 676 days in vitro respectively. After 980 days in vitro and 59 serial subculture generations, cells of Exp. 19 had not exhibited a stabilized mode through successive generations but does show an accumulation of abnormal chromosomes which may be termed markers at this time. In later generations there has been a narrowing of the range of chromosome numbers. In Exp. 26 a second generation culture of 697 days had a hypodiploid-diploid bi-mode at 39-40 but with only 55% of the population in the diploid range. Of the cells examined from Exp. 26, 19% contained

minute chromosomes. Examination of the cells from one of the transplanted tumors produced by cells from Exp. 26 showed that the modes were hypodiploid with 39 chromosomes per cell at 549 total days from explantation and hyperdiploid with 43 chromosomes per cell at 658 days. Chromosomal abnormalities such as biarmed chromosomes (metacentrics, submetacentrics, and subtelocentrics) were not noted in the cells of two of the experiments (32 and 26). The latter did not become established cell strains but grew enough for some tumor tests. Biarmed chromosomes were also not noted in Exp. 33, which did become established and after 900 days in vitro gave tumors. Biarmed chromosomes were also noted in the first sample and frequently in all subsequent samples of cells in Exp. 19 which became established in vitro. For purposes of contrast, cells of Exp. 19 and 33 both gave tumors but both were not characterized by biarmed chromosomes.

Significance: This is, to our best information, the first time that freshly explanted tissues from normal tissue have been grown in a chemically defined medium in sufficient amount and in good enough quality to be used as a continuing biological tool in any study before, during, and after neoplastic transformation. While the incidence of tumor production is very low and the time required for the cells to transform (ranging from 169 to 411 and more recently 900 days) does not appear to be as reproducible as cells grown in horse serum supplemented medium; the final results, after all the test animals have been autopsied and the implants examined histologically, still await evaluation. In the five current experiments, cell re-growth in the cultures after removal of cells for implantation is sufficient to allow repeated use of the same donor cultures as a source of high quality tissue cells for a statistically significant number of intraocular implants, greatly enhancing the validity of results. Though progressively growing tumors have been obtained by one other investigator using chemically defined medium, small amounts of commercial insulin were included in the culture medium and the original isolation was accomplished with the undefined enzyme preparation, pangestin. Tumor tests were made by the other investigator only after the cells began to grow rapidly, whereas ours were initiated soon after explantation. Thus we were able to pinpoint time of neoplastic conversion. It was demonstrated that cultures could be maintained in chemically defined medium without chromosomal structural abnormalities such as biarmed chromosomes, but these cells were not growing rapidly and were not exposed to serial subculture. With rapid serial culture the abnormalities increase.

The fact that perfectly normal, highly differentiated tissues were seen in intraocular implants made after more than a year's culture in vitro is especially significant for the following reasons:
1) The culture of mouse embryo cells in chemically defined medium NCTC 135 apparently favors the maintenance of an undifferentiated fibroblast cell type (except for the epithelial cell type which appears to be differentiated germinativum) which is capable of differentiating into the various connective tissues in the anterior chamber of the eye.
2) Minced embryos (never cultured in vitro) and minced embryo cells carried for shorter periods in vitro produce a larger variety of tissues in the eye than those cultured for longer periods in vitro where the differentiation is limited to three or four specialized types of connective tissue. Apparently,

the ability to differentiate is narrowed to just connective tissues with increased culture in vitro.
3) The growth and differentiation of normal tissues in the anterior chamber of the eye indicates the absence of rejection by the host and validates the assumption by this laboratory that the anterior chamber of the eye is indeed an ideal site for malignancy tests since even normal tissue is not rejected at this site, though it does not progress to formation of transplantable tumors.

Proposed Course of Study: The follow up on tumor production and check for presence of leukemia will be completed, data consolidated, compared, and prepared for publication. Any line found to be non-neoplastic will be used for transformation studies.

Project Description (b): Comparative studies on the malignant transformation in vitro of freshly explanted C3H mouse embryo cells in chemically defined medium NCTC 135 supplemented with either horse serum or fetal calf serum, and the behavior of sublines made by switching the serum supplement.

Objective: To investigate and elucidate the nature of the neoplastic conversion and mechanism by which C3H mouse embryo cells undergo spontaneous neoplastic transformation or conversion in vitro. An attempt will be made to correlate any chromosome alterations with the onset of malignant transformation.

Methods Employed: Twelve to thirteen day old C3H/HeN embryonic mice were minced and cultured in medium NCTC 135 supplemented with 10% v/v of either fetal calf serum or horse serum. Establishment of cultures in T-flasks was carried out by routine culture techniques. Intraocular injections into the mouse strain of origin were made with aggregates of cells produced by the above cultures after various periods of cultivation. To prepare the chromosomes, cultures in the log phase of growth were treated with 0.03 ml of 0.2 gamma solution of Colcemid per ml of culture medium for from 4 to 13 hours at 37.5°C to accumulate cells at the metaphase. The cells were harvested by means of the action of versene and trypsin or scraping. Varidase was used to aid in the separation of clumps into individual cells after trypsin action. Sodium citrate or distilled water was used for the hypotonic swelling. A fixative of acetic acid and methanol was used. A portion of the cell suspension was air-dried on the microscopic slide and stained with aceticorcein. The above technique is similar to that described by German et al. in J Nat Cancer Inst 32: 681-707, 1964.

Sublines were made from some of the parent lines after various periods of cultivation in vitro by changing the serum used to supplement medium NCTC 135. All other conditions were identical for the parent line and sublines. Chromosome analyses were made before and after switching serum.

Major Findings: Without exception independently established strains of mouse embryo cells and, as reported earlier, 3 strains of 2-day-old C3H mouse kidney cells, when cultured in medium NCTC 135 with 10% v/v horse serum, produced tumors after 98 days and predominantly before 200 days. In contrast cells cultured in medium supplemented with 10% v/v fetal calf serum failed to produce tumors or produced them from cells in vitro in this serum supplement after

a longer time than the cells in horse serum. Pairs of lines from a common pool may show the absence of leukemia virus, but the line on horse serum still shows conversion. Usually if the time on fetal calf serum has not been in vitro an inordinately long time, it requires the same period of time when switched to horse serum to show evidence of conversion; as one example this was 182 days. If in fetal calf serum for over 400 days before being switched to horse serum, then the time on horse serum has extended to approximately 300 days. Cells converted in horse serum continued to produce tumors when transferred and cultured in fetal calf serum. Cells exposed as short a time as 15 days to horse serum and subsequently switched to growth in fetal calf serum still showed neoplastic conversion but they were delayed materially in the conversion in at least one series. Perfect correlation between length of time in horse serum and subsequent duration of exposure to fetal calf serum to time of conversion and ability to produce tumors was noted in one study, but this finding has not always held firmly in other studies.

Non-neoplastic long-term strains in medium with fetal calf serum are predominantly aneuploid with respect to chromosome numbers. Aneuploid cells in vitro have been considered by some investigators as being malignant.

Mouse embryo cells cultivated in fetal calf serum supplemented chemically defined medium retained their diploid mode longer than those in horse serum. Non-diploid modes were no more stabilized or well defined in fetal calf serum than horse serum. In those cell strains which became neoplastically transformed, irrespective of species of serum supplementation, there was a trend toward a marked increase in percent of cells with abnormalities at about the time of neoplastic transformation. There was a definite increase in percent of cells with breaks, minutes, and biarmed chromosomes after the cell strains became neoplastic. Cell strains in fetal calf serum had a lower percentage of cells with breaks, minutes, and biarmed chromosomes than those in horse serum during the initial 9 months in culture irrespective of neoplastic transformation. During the total pre-neoplastic periods those strains on fetal calf serum that transformed averaged less abnormalities than those on horse serum although not having undergone neoplastic transformation. Long-term strains on fetal calf serum had as high or occasionally a higher percent of cells with abnormalities than those on horse serum.

Cells switched from growth in fetal calf serum to horse serum at various times such as 15, 60, and 90 days revealed that the earlier the switch the greater were the modal, chromosomal number alterations, and abnormalities. Abnormalities compared to control maintained on fetal calf serum and later switches of later date at equal times in vitro. The longer the time of cultivation in fetal calf serum before switching to horse serum medium, the less drastic were the alterations. In the timed switches of cells from horse serum to fetal calf serum the converse was true with one exception. The earlier the switch, the less the alteration compared to control on horse serum and later to switch of later date at same age. Alteration compared to control on horse serum and later switches of later date at equal times in vitro. Nevertheless, there is great variation of chromosomes and morphology in one serum supplement even between two separate lines. All data on effect of switches of serums and relation to onset of neoplastic transformation are being correlated for a manuscript in preparation.

Significance: It appears that fetal calf serum supplementation of chemically defined medium provides a more stable environment than horse serum for the establishment of cell strains in so far as the chromosomal alterations that take place during this period are concerned. Unknown stabilizing factor(s) in fetal calf serum appear to delay the shift of chromosome number and mode from diploidy and the occurrence and prevalence of abnormalities. Established cell lines may be chromosomally characterized as aneuploid or heteroploid and have a very high level of abnormalities and yet still not be neoplastic as evidenced by failure of cells to produce tumors when implanted into the anterior chamber of the eye in isologous hosts.

The fetal calf serum appears to have either selected for, stimulated the growth of, or stabilized the environment in some way for advantageous maintenance of cells less chromosomally alterable. Thus, to reiterate, for the maintenance of cells with the least alterations, fetal calf serum is a better serum supplement than horse serum.

The consistency of the data on the effect of fetal calf serum relative to transformation gives us working tools to explore the mechanism at the molecular level of neoplastic transformation in vitro (see NCI Serial No. 3234). We, for the first time, seemed to have uncovered a factor in the medium used for cells in vitro that has an apparent effect on neoplastic transformation. We have shown that aneuploid cells are not necessarily neoplastic, and Sanford and her coworkers have confirmed these findings. We have found that once cells were neoplastic continued cultivation in fetal calf serum and injection of the cells with the fetal calf serum still produced tumors, so that an immune response of the host does not explain the effect of the fetal calf serum; the neoplastic state of the cells was not reversed by exposure to fetal calf serum. The data are strong enough to pose working concepts some of which are already under exploration (NCI Serial No. 3212, project 2, and NCI Serial No. 3211), such as the effect of fractions of the active serum. The possibility that the fetal calf serum may be having a repressor-like action is another working concept. This concept can be tested further with the 5009 cells initiated in chemically defined medium and never in any serum. To date any injections of strain 5009 have been negative. Another way to test the concept is by way of the strain 4823 which has been over 4 years in vitro and has not produced any tumors. Sublines of it in chemically defined medium injected into animals offer a means to see if release of these cells from fetal calf serum will release them from any repressor-like action the fetal calf serum may have and tumors will result.

Proposed Course of Work: Since it has been noted that even 15 days exposure to horse serum resulted in the cells being neoplastically transformed, studies were instituted to determine the minimum time of exposure to horse serum for the conversion to take place (see NCI Serial No. 3233). A manuscript is in preparation on the complete analyses of the effect of switching serum both on the neoplastic conversion and the chromosomes.

Project Description (c): Studies on the spontaneous neoplastic transformation of a non-neoplastic long-term cell line cultivated in medium NCTC 135 supplemented with 10% v/v fetal calf serum.

Objective: To attempt to induce the neoplastic transformation of a non-neo-

plastic C3H/HeN mouse long-term cell strain, NCTC 4823, cultivated for over 1000 days in medium NCTC 135 supplemented with 10% v/v fetal calf serum by changing the serum supplement to 10% horse serum.

Methods Employed: After these cells had been growing in medium containing fetal calf serum for over 1000 days without undergoing a malignant transformation, the medium on several cultures of these cells was changed to horse serum instead of fetal calf serum. The medium was changed on cultures that has been growing in the fetal calf serum medium for 1018, 1100, 1116 days and subsequent times thereafter. In initiating some of the new cultures after 1147 days and thereafter, fluid from horse serum cultures initiated at 1018 days was added to the new cultures after centrifuging the supernatant for 20 minutes at 2000 RPM. The reason for this will be explained in the results below.

The horse serum cultures were tested at various intervals for malignant transformation by either intraocular or intramuscular implantation into C3H/HeN male mice. Any tumors resulting from these injections were transplanted and sectioned and stained for histological examination.

Chromosome studies were made on the horse serum grown cells as well as tumors which resulted from the injections. For this information see the project report of Dr. John T. Mitchell, NCI Serial No. 3233. Cultures and some tumor material was also sent to Dr. Patricia Farnes, Rhode Island Hospital, for histochemical investigation, see NCI Serial No. 3212, project 3, for these procedures and findings. Sample cultures are routinely sent to Dr. Janet Hartley for examination for detection of murine leukemia virus by the complement-fixation method.

Major Findings: The first switch to horse serum containing medium was made when the cells had been in culture for 1018 days. After the cells had been in the horse serum medium for approximately 2 weeks, 2 cultures each showing distinctly different morphological characteristics in light microscopy became apparent. One culture seemed to be composed of mainly fibroblast-like cells like the fetal calf serum cultured cells although growth of the new horse serum cells was very sparse. In 4 weeks time this subline died out. The other subline of the horse serum cultivated cells began to show many more smaller round cells. These round cells appeared to proliferate rapidly and covered the floor of the flask. The adhesion of these cells to the floor of the flask was not great and a large number of them floated free in the supernatant fluid. An attempt to implant these cells in the anterior chamber of the eye of host C3H/HeN male mice to test for neoplastic transformation was made after the cells had been growing in the horse serum containing medium for 60 days, but was unsuccessful since the cells grew singly or only in small clumps and were unsatisfactory for the intraocular implant technique.

When the cells had been in horse serum medium for 95 days, 4 C3H/HeN male mice irradiated with 425 r were injected intramuscularly with approximately 5 x 10^6 cells. Papable tumors were obtained in 4 days. Sections of a tumor were prepared for histological examination by Dr. Thelma B. Dunn. Cultures injected into mice at later dates all produced tumors in the host animals. The tumors in all animals came up in about 4 to 6 days and became necrotic and quite vascular several days after becoming palpable. Histological slides of tumor

sections have been inconclusive thus far but it appears that the tumor is of a tymphoma type.

Chromosome studies have been made on the fetal calf serum and horse serum cells and on tumor tissue. Chromosome studies have indicated that there are many diploid cells in the horse serum culture compared with the number of diploid cells in the fetal calf serum cultures. For further chromosome data, see the project report of Dr. John T. Mitchell, NCI Serial No. 3233.

Three months after initiation of the first horse serum cultures 2 more lines were set up in an effort to duplicate the results obtained after the first switch to horse serum. Neither of these cell strains have grown like the first switched line in that there has been no prevalence of small round cells. Intramuscular injections made into C3H/HeN mice irradiated with 425r have not produced tumors as yet after over a year. Chromosome analyses indicate that these cells are not unlike the original fetal calf serum cultures.

Culture flasks of all three switched lines have been sent to Dr. Janet Hartley for testing for the presence of murine leukemia virus by the complement-fixation test. Only the culture switched to horse serum the first time gave a positive test. In another attempt to produce a horse serum culture like the first one, another fetal calf serum culture was switched to horse serum and 1 ml supernatant fluid from the original horse serum culture was added after centrifugation for 20 minutes at 2000 RPM to the new culture. In 4 weeks time the new culture appeared like the original switched line, containing mostly small round cells. These cells also produce tumors and contain leukemia virus particles and have chromosomes like the first horse serum line.

However, by this method there was the possibility that we had transferred some cells from the supernatant and that they eventually took over the culture. To avoid this possibility we set up new cultures by again centrifuging a suspension of one of the tumor producing lines and filtering the supernatant by Selas 03 and Millipore before putting it in the new cultures. Cultures receiving the filtered supernatant were grown in medium containing horse serum and in medium containing fetal calf serum. All of these cultures were shown by electron microscopy and/or the CF test to contain virus particles (see NCI Serial No. 3225), but none of the cultures developed a population of round cells and did not produce tumors when injected into isologous hosts. Also, virus material was isolated from tumors produced by the first horse serum line and new cultures containing this virus were set up again in both horse and fetal calf serum containing medium. Again the virus was detected in the cultures although no tumors have been produced by these lines and the cultures remained predominantly fibroblast-like. Generally the chromosomes of all these cultures were primarily like those of the original non-neoplastic cells grown in the fetal calf serum containing medium. For more detailed chromosome information see NCI Serial No. 3233.

Significance: It would appear from these studies that the virus isolated from the tumor producing line is not alone responsible for the neoplastic transformation of this cell line. It is possible, of course, that by filtering the virus some of its activity was lost. This possibility will have to be further

investigated. It is of further significance that we have been unable to transform these cells simply by the use of horse serum in the medium rather than fetal calf serum. There is the possibility that due to the extremely long time that these cells have been cultured in vitro in fetal calf serum containing medium without becoming neoplastic the exposure time to horse serum needed to bring about neoplastic transformation has been significantly increased.

Proposed Course of Study: The cultures grown in horse serum containing medium which do not have the virus will be carried and injected periodically to see if eventually these cultures will undergo neoplastic transformation. A substrain of these cells is now growing in chemically defined medium. These cells will be tested by the same methods mentioned above in order to determine if serum may have some preventive effect on neoplastic transformation when filtered virus is added to the cultures. Chromosome analysis and continuing tests for the presence of leukemia virus will be made on these cells.

Project Description (d): Studies comparing the effect of adult and fetal serum on the neoplastic transformation in vitro.

Objectives: To determine if the property of greatly delaying or preventing the transformation is a property of fetal sera in general or a property peculiar to fetal calf serum alone.

Methods Employed: Two separate experiments with five lines each were initiated from a common pool of minced embryos cells. These lines were then established and cultured on NCTC 135 supplemented with 10% horse, fetal horse, calf, or fetal calf sera (6-month gestation and 3-month gestation). These five lines have been subjected to periodic tests for malignancy and chromosome analysis.

Major Findings: This study is in final stages of completion and a manuscript is in preparation. The results to date of periodic chromosome analysis are presented in NCI Serial No. 3233. Cells in sera from fetal calves of 2 to 3 months gestation became neoplastic after 231 days in vitro. Cells in sera from fetal horses became neoplastic by 166 days. The cells in calf serum and gelding horse serum became neoplastic at approximately the same time in vitro. The cells in fetal calf serum from fetuses 5 months showed delay over the time the cells in horse serum underwent neoplastic transformation.

Significance: At least with the two species of fetal serum available to study, fetal serum irrespective of species is not a factor in the delay of neoplastic conversion and for that matter it appears that, even with fetal calf serum, for the serum to have bearing in the delay of neoplastic conversion of cells in vitro, serum from only a certain aged fetus appears to be active in this delaying phenomenon since serum from younger fetuses and from newborn calves does not possess the quality. The effect of fetuin, the most readily obtained characteristic fraction of fetal calf serum should be evaluated from data presented in NCI Serial No. 3213. In general, one is tempted to theorize that fetal calf serum develops, as the embryo develops, a concentration of a factor of a repressor-like nature capable of delaying neoplastic conversion, but this factor is lost after birth. The data presently being assembled on adaptation of strain 4823 to serum-free medium hopefully will shed some information on

the merit of this theory on the repressor-like effect of fetal calf serum. Should the several lines of 4823 (see NCI Serial No. 3212, project 1) adapted to chemically defined medium be shown to have become neoplastic, it would give some credence to the theory of the repressor-like action.

Proposed Course of Study: This particular project is being written up but the concept arising from this study of a possible repressor-like action of the fetal calf serum will be evaluated and the merits of becoming engaged in a necessarily long-term study to evaluate the concept will be seriously considered.

Project Description (e): Studies on the effect of various sera fractions of horse and fetal calf serum on the neoplastic transformation.

Objectives: 1) To determine if a fraction of horse serum is or contains the inducer of the neoplastic transformation; 2) or whether a fraction of the fetal calf serum contains a repressor-like substance; and 3) to assess the implications of the results in relation either to carcinogenic action, repressor-like action, or cell selection.

Methods Employed: Two fractions have been obtained from each of the two sera. (See NCI Serial No. 3234). Further fractions of the large molecule fraction are in preparation. All fractions ultimately are to be used for cultivation of a common pool of fresh C3H mouse embryo cells to test the objectives by following ability to produce or supress neoplastic conversion of cells in vitro.

Major Findings: This study could be 2 to 3 years in duration before all data are in on the long-term cultivation of cells and the necessary long-term holding of injected animals; therefore, much preliminary work has had to be carried out prior to the major long-term study. Methodology was worked out for fractionating the sera and for appropriate sterilization of the sera fractions so both toxicity would not interfere and activity would not be lost. The fractions have been tested with regard to the parameters above on cells similar to those to be involved in the long-term study and have been found to be biologically satisfactory. The long-term study on all fractions, combinations, i.e., reconstitutions thereof, and the serum from which the fractions have been made has been initiated on the cells.

Significance: The significance to the objective must await the results of injection of the cells. However, it is most important to have learned that the fractions we are using are not toxic to the cells. Any commercial fractions employed previously have been either immediately toxic or have showed delayed toxicity and have prevented carrying out studies. Should any fraction have a specific biologic action in producing the neoplastic conversion, it will be further fractionated to attempt to cut out clearly the active component.

Proposed Course of Study: To complete study as proposed and to further fractionate any fraction giving information toward obtaining the final objective.

Project Description (f): Studies on the transformation with a chemical carcinogen (7,12 Dimethylbenzanthracene) of NCTC 4823, a non-neoplastic line from minced C3H mouse embryos.

Objectives: To transform a non-neoplastic line, NCTC 4823, by means of a chemical carcinogen, 7,12 Dimethylbenzanthracene (DMBA). Upon transformation, biochemical, histochemical, and cytogenetic studies, etc., are to be made in order to determine the differences between the neoplastic and non-neoplastic lines at the molecular level.

Methods Employed: Non-neoplastic NCTC 4823 cells were treated with a total of 7.5 gamma of DMBA in NCTC 135 + 10% fetal calf serum over a period of 10 days with the last treatment remaining 24 days for a total of 34 days the cells were in DMBA. A limited population survived this treatment. These cells were fluid changed with NCTC 135 + 10% fetal calf serum for about six months; limited cell division took place. All free floating cells were transferred into a flask with a sheet of cellophane covering the floor to help retain the cells. A small but significant increase in cell number immediately resulted. The concentration of the fetal calf serum in the medium was increased to 20%, and the line began to recover rapidly. Continued fluid changing and subculturing using the above medium and method resulted in a line, now termed NCTC 6049, that could be cultured with relative ease although the growth was still slightly inferior to that of the control, NCTC 4823. After culturing the line on cellophane sheets for about four months, a few flasks were successfully subcultured to ordinary glass flasks. Due to the possibility that the two lines, on and off cellophane, may be different, they were carried separately and the line removed from cellophane termed NCTC 6182. The chromosome methods have been described by J. T. Mitchell, NCI Serial No. 3233. The cultures are now rapidly growing and injections have been made. Leukemia tests are under way.

Major Findings: In so far as the present study has developed, the non-neoplastic 4823 cells subjected to 7,12 DMBA were severely affected by the DMBA. The cells after DMBA treatment were fibroblast-like and/or round cells. Only a small number of cells of the original population survived and the growth rate was low. This would initially suggest that cell selection from the treated 4823 population had occurred. The chromosome analyses (NCI Serial No. 3233) showed that the population evolved from the cells treated with DMBA appears to have no progenitor in the original 4823 cells. Thus there appears to be as the most conservative opinion a direct pharmacologic action of the DMBA on the cells,--i.e., more venturesomely, possibly a direct effect on the hereditary material of the cells. Another observation is that the cells are greater acid producers. Compared with the cells untreated with DMBA, i.e., the 4823 controls, the alkaline phosphatase activity is reduced by one-half to two-thirds, the arginase activity is reduced a little less than half. However, the acid phosphatase activity is almost twice as great as that of the control cells. By electron microscopy no virus particles have been observed.

Significance: Reports in the literature on the action of chemical carcinogens in vitro have made a point that chemical carcinogen on non-neoplastic cells destroys all the cells. Other studies in the literature have reported on the effect on strains that have a low incidence of tumors even without treatment with carcinogens. Further, reports in the literature point out the difficulties

of other investigators in maintaining non-neoplastic cells at all in vitro, hence there has been a scarcity of control cells to evaluate the significance of effect of chemical carcinogens. Other reports where fresh tissue cells have been grown point out that the carcinogen may effect neoplastic conversion somewhat earlier than it occurs spontaneously. The present study reported here is unique in that the cells used for this carcinogen study have remained non-neoplastic after four years of cultivation in the fetal calf serum and being subjected to continuous testing and frequent chromosome analyses. The line seems eminently suited for this type study where it is important to evaluate treated cells in comparison with untreated controls where injections to test neoplastic transformations have been continuously made and in more than adequate numbers with high cell populations in irradiated mice to demonstrate that the line is non-neoplastic. Furthermore, by electron microscopy the 4823 line has revealed no type C particles (W. T. Hall, NCI Serial No. 3225). From tests for complement-fixing mouse leukemia antigens, the 4823 cells are negative. All these parameters at least make this line of cells the best in the laboratory and for that matter the best known to us from any source for studies on the mode of action of a chemical carcinogen. Depending on the outcome of the injections only recently made on both the carcinogen treated cells and cells in chemically defined medium (NCI Serial No. 3212, project 1) will decisions be made on the advisability of expanding the study with the cells presently used in this study or the possibility of carrying out a similar study on the sublines adapted to serum free chemically defined medium. Should the latter idea prevail, possible interfering factors in serum would be removed to yield cleaner data. This has been the primary reason the laboratory has devoted so much time to adaptation and/or initial isolation of cell lines to serum free medium. It must also be clearly recognized, and the proof sought before such a study is initiated, that the possibility exists that the use of fetal calf serum is a factor in controlling stabilization in the normal direction and cells adapted away from it may become neoplastic and would not be useful for such a study on action of the carcinogen.

These cells treated and untreated with DMBA present a second pair (NCI Serial No. 3234) to determine if there is a difference between non-neoplastic and neoplastic cells and tumors from the latter in their RNA methylase capacity. One can study the relationship of nucleic acid methylation to oncogenesis where the non-neoplastic control cells can be maintained for comparison throughout the study (NCI Serial No. 3234). Thus one would be answering the question of whether this is a quality distinguishing normal from tumor cells derived by chemical carcinogens as well as by horse serum exposure. Such studies require large numbers of cells and this accounts for our continuing search (NCI Serial No. 3211, 3220) for means to attain large amounts of cells under controlled conditions.

It is recognized that rat cells are 1) chromosomally more stable than the mouse and 2) are shown to undergo neoplastic conversion even though they produce a lower percentage of tumors. Nevertheless, the high degree of inbreeding of the C3H mouse, the wealth of general information on the colony, and the amount of information on this particular cell line, 4823, weighs heavily in favor of this C3H mouse line for the type study under way. Even the continuous monitoring of chromosomes becomes less formidable if automated methods (NCI Serial No. 3233)

are worked out. Another advantage in so far as we can ascertain by methodology available is that the control cells 4823 are virus free to rule out contaminating tumor viruses as causes of "spontaneous" neoplastic conversion.

Proposed Course of Study: The properties of the untreated cell strain and the DMBA treated derivative will be followed. Under significance, some of these have been pointed out. Cytogenetics, ultrastructure morphology by brightfield and EM, and cytology will be compared. Tumors, if they occur, will be followed in the same way. Glucose uptake will be fully explored, as well as lactic acid production. Qualitative and quantitative differences in enzyme activity will be determined. All known parameters will be explored. New indicators will be sought with some consideration of cytology, particularly those indicators used in human cancer diagnostic cytology. Structural differences at the EM level will be emphasized both with regard to cell membrane, ribosomes, and cytoplasmic inclusions. For example, the question is asked has there been an increase in the smooth endoplasmic reticulum during DMBA treatment; this might represent a characteristic in the cells of transformation by the carcinogen when the cells try to repair damaged detoxification mechanisms. The question will be asked whether isolated ribosomes from the transformed cells are refractory to stimulation by synthetic mRNA. The media exposed to treated cells and untreated cells could be exposed to "fingerprinting" by gas-liquid chromatography to detect and identify components of the chemical mixture after neoplastic transformation as well as medium from the control. This type approach has been used not only on metabolic products but also on bacteria and viruses. The non-neoplastic cells and neoplastic cells and medium from them might be easier to fingerprint and subtle metabolic differences could be thus determined.

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Evans, V.J.: Spontaneous neoplastic transformations in C3H mouse cells in vitro; presented at International Conference of Tissue Culture in Cancer Research, Tokyo, Oct. 21-22, 1966; published in Monograph entitled "Cancer Cells in Culture" March 1968.

Honors, Awards, and Other Activities: V. J. Evans.

1. Served as Session Chairman and Abstractor on Characteristics of Cell Culture Systems at Working Conference on Cell Culture for Virus Vaccine Production organized by Division of Biologics Standards, NIH, 1967. Monograph of Proceedings to be published by NCI.

2. Presented seminar on Spontaneous Neoplastic Transformation at Laboratory of Human Genetics, Cornell University Medical School, May 1967.

3. Serving as Chairman, Publicity for Fifth Annual Meeting for Society for Cryobiology.

4. Serving as Consultant to Tissue Bank, National Naval Medical Center.

5. Serving as Member, Biology Panel, Interagency Civil Service Review Board.

6. Serving as Member on Resources and Logistics Segment of Special Virus Leukemia Program.

7. Serving as Member, Special Fellowship Board, National Cancer Institute, NIH.

8. Serving as Member Ad Hoc Committee of Tissue Culture Association for publication of Bibliography of Tissue Culture.

9. Serving as Organizing Chairman, 1970 Annual Meeting, Tissue Culture Association in Washington, D.C.

NCI Serial No. 3212, Project 3 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project 3 Title: Miscellaneous collaborations in tissue culture with other organizations.

a. The study of the structure properties and metabolism of nucleic acids and nucleoproteins of C3H mouse embryo cells in vitro.

b. Comparison of cytologic and histochemical properties of a highly heterogeneous non-neoplastic C3H mouse cell population in vitro with its neoplastic derivatives.

c. Cytologic and histochemical properties of a common pool of non-heterogeneous population of cells on either fetal calf serum or horse serum correlated with continuing changes resulting from transfer to cultivation in horse serum.

d. Isolation of leukemia virus from a strain of cells isolated in chemically defined medium for determination of ability to neoplastically transform cells in vitro.

Principal Investigator: V. J. Evans

Other Investigators: W. F. Andresen, F. M. Price, and J. T. Mitchell

Cooperating Units: a. Dr. J. Rotherham, Series 397, and Dr. W. Schneider, Nucleic Acid Section, Laboratory of Biochemistry, National Cancer Institute

b. Dr. Patricia Farnes and Dr. Barbara Barker, Rhode Island Hospital, Providence, Rhode Island

c. Dr. Barbara Barker, Rhode Island Hospital, Providence, Rhode Island

d. Dr. W. T. Hall, Series 3225, General Biology Section, Laboratory of Biology, NCI, and Dr. Janet Hartley, Laboratory of Infectious Diseases, NIAID

Project Description (a): The study of the structure, properties and metabolism of nucleic acids and nucleoproteins of C3H mouse embryo cells in vitro.

Objective: To determine if there is any change in the structure, properties and metabolism of the nucleic acids or nucleoproteins of the cultured cells of the C3H minced mouse embryo as they grow continuously in vitro as neoplastically transformed cells.

Methods Employed: The cells used in these experiments are neoplastic C3H mouse fibroblast-like cells from embryo mince. The cells have been adapted to growth in completely chemically defined medium. Considerable preliminary work has been necessary to adapt the biochemical techniques to the limited amount of available in vitro material. Presently modifications are being used for the isolation of deoxynucleosides from the medium by column chromatography according to the method of R. N. Hall. DNA from the cells has been prepared by phenolic extraction according to a modification of the methods of Kirby and Kay and the nucleosides from the DNA have been obtained by digestion and isolated by paper chromatography. The determination of amounts and specific activities of medium and DNA deoxynucleosides is based upon a microbiological assay. Various C^{14} labelled nucleic precursor compounds were added to the medium to follow their utilization.

Major Findings: Cells which were grown in chemically defined medium NCTC 135 utilized labeled thymidine or deoxycytidine for DNA-thymidine synthesis to a greater extent than they did cytidine, uridine, deoxyuridine, or orotic acid. The specific activity ratios of DNA-deoxycytidine to DNA-thymidine for these precursors were: deoxycytidine, 1; cytidine, 5; uridine, 10. When the folate content of the medium was increased ten-fold, there was a greater utilization of cytidine for both DNA-pyrimidines and to a lesser extent an increase in the utilization of deoxycytidine, deoxyuridine, and uridine for DNA-thymidine. A cell strain derived from the original was grown in medium containing the ten-fold higher level of folate for 13 transplant generations and then subjected to tests similar to those for the original strain. For these cells there was a marked increase in the extent of utilization of cytidine, uridine, and deoxyuridine for DNA-thymidine and the specific activity ratios of DNA-deoxycytidine to DNA-thymidine decreased: deoxycytidine 0.3, cytidine 1, uridine 1. The extent of utilization of orotic acid remained comparatively low. A manuscript on this study has been submitted for publication.

Significance: This work has been carried out in an effort to gain additional knowledge bearing on the questions: (1) What environmental conditions are necessary for continued growth of neoplastic mammalian cells; (2) In what manner do these conditions influence cell division; (3) What changes occur in the synthetic pathways of metabolites which are essential to cell division for cells which have undergone neoplastic transformation. In this study we have presented data that indicate that the folate content of the culture medium might be one of the primary factors necessary for these cells. This study may take on special significance if similar studies involving non-neoplastic cells can uncover differences in cell division and growth of non-neoplastic cells.

Proposed Course of Work: To examine some of these aspects of metabolism of nucleic acid precursors in cultures of non-neoplastic cells. It would be particularly desirable to conduct such studies over a period of time when the cells are grown under conditions which permit prediction of the time of neoplastic transformation or where the studies were carried out on cell lines that are non-neoplastic but can be converted at will by known agents such as a chemical carcinogen. (See NCI Serial 3212, project 2).

Project Description (b): Comparison of cytologic and histochemical properties of a highly heterogeneous non-neoplastic C3H mouse cell population in vitro with its neoplastic derivatives.

Objective: To determine the morphologic and histochemical properties of strain 4823, a long-term non-neoplastic strain, compared with its neoplastically transformed sublines.

Methods Employed: Cultures of the non-neoplastic strain 4823 cultured over 1400 days in fetal calf serum have been compared with 3 derivative lines switched to growth in horse serum containing medium in order to transform cells neoplastically. These are being studied continuously. The morphology in the strain and sublines has been studied with Wright-Giemsa stained coverslip preparations. The following histochemical studies have been performed on some lines and are in progress on others: acid phosphatase, alkaline phosphatase, glycogen, phosphorylase, and the following dehydrogenases: succinic, isocitric, lactic, glucose, dehydrogenase, glutamic, and B glucuronidase. These were selected to indicate activity of the Krebs cycle, glycolytic pathway, hexose monophosphate shunt, and fat metabolism. The following methods for example were used: acid phosphatase (Barka-Goldberg), alkaline phosphatase (Gomeri Azo), glycogen (PAS and diastase digestion), and dehydrogenases (Barka Anderson), B glucuronidase (Hayashi).

Major Findings: Morphologically in the parent strain 4823 five cell types were noted: A fibroblast-like population and four distinct categories of small cell types. The small cells were distinguishable from each other by both general morphology and differences in size and shape of nucleus and cytoplasmic staining. Further distinction was revealed with the data from histochemistry. Most recently, some decline of type I cells is becoming evident. The three sublines obtained have been distinguished both from the parent line and each other by the same parameters. One subline 5645 so far has given tumors which appear to be lymphomas. The population of cells giving rise to these tumors has only two cell types instead of the five in the parent line. To date these tumors are not yet completely analyzed for comparison of morphology and histochemistry with the cultured cells from which they were derived and with the original strain of origin. One of the two types of cells in the transformed line is fibroblast-like and is morphologically and histochemically the same as the fibroblast-like cells in the parent strain 4823. The second type belongs to the small cell type of the parent strain but is not identical with any members of the other four cell categories of the parent strain. In general morphology, the second type most closely resembles the type 1 cells of the parent strain but can still be clearly distinguished on the basis of shape and content of nucleus and also on the differences in cytoplasmic staining and inclusions.

Besides quantitative differences in the histochemistry of the small cells of the parent strain and the transformed strain, one qualitative difference exists. The population transformed by horse serum and producing round cell tumors has acquired alkaline phosphatase activity not present in any of the five cell types of the parent strain. Loss of lysosomal enzymic activity, changes in lipid synthesis, special physiologic behavior such as reticulin formation, rosette formation, and peripolesis have also been noted. Results from two other sublines of strain 4823 obtained in the same way as line 5645 by cultivation in horse serum are showing still different morphology and histochemistry distinguishing them from each other and from their parent. The morphologic differences are the greatest; the histochemical differences for the most part are at a quantitative level. The two newer lines like the parent line do not have alkaline phosphatase and are therefore unlike their sister line 5645. Development of acid phosphatase activity and B glucuronidase activity are examples of apparent differences in parent strain and first tumor producing line from subsequent lines in horse serum of the same parent line. One of these 5716 is showing some loss of Type III cells (See manuscript under serial No. 3212, project 1). The two newer lines cultured in horse serum are being tested for tumor production to determine universality of the neoplastic transformation. To date the two so-called newer lines have not given tumors, but histochemically and morphologically 5736 cells are showing some characteristics indicative of neoplastic conversion; therefore the cell injections are being carefully and frequently observed to determine if these cell changes are indicative of neoplastic conversion of the general classes already used in diagnosis of uterine, lung, and stomach cancer in humans. A manuscript has been published on the work to date. (See serial No. 3212, project 1.)

Significance: Morphologic and/or metabolic differences are showing up between cells in the mixture of cells in the control non-neoplastic cultures. Immediately this seems to characterize and categorize the cells of the control population. Some distinctive differences are showing up in the neoplastically transformed line. All the differences between the parent normal strain and at least one of the lines subjected to horse serum as noted under Major Findings suggest profound metabolic adaptations were associated with the transformation. If these can ultimately be shown to serve as true "handles" to define biochemical and physiologic manifestations associated with the transformation process, then morphology and cytochemistry of cells in vitro will be valuable in defining the mechanisms of neoplastic transformation. Some of the parameters being employed with the cells in vitro are of the same general classes already used in diagnosis of uterine, lung, and stomach cancers in humans.

Proposed Course of Work: The detailed morphologic and histochemical properties of the parent line are being continuously studied parallel with the three daughter sublines that are converted or subjected to horse serum. Where appropriate, cultured tumor lines of any of these sublines will be studied. Correlations of general morphology and cytochemistry on parent strain and all sublines will be made with complement-fixation tests, electron microscopy findings on presence of leukemia virus, and tumor production, since all of these are negative so far for presence of virus. Hopefully, specific trends may be established in the histochemical changes observed as cells transform from

normal to neoplastic cells that will reveal possible molecular inducers of the neoplastic transformation.

Project Description (c): Cytologic and histochemical properties of a common pool of non-heterogeneous population of cells on either fetal calf serum or horse serum correlated with continuing changes resulting from transfer to cultivation in horse serum.

Objectives: 1) To determine the transitions in morphology and histochemistry in a common pool of non-heterogeneous strain of C3H mouse cells grown in either horse serum or fetal calf serum and when an aliquot of the latter strain is switched to cultivation in horse serum; 2) To attempt to correlate the above changes and variations with results of the in vivo demonstration of neoplastic transformation by horse serum; 3) To confirm findings of preceding study (b) for common characteristics of normal and neoplastic cells.

Methods Employed: A common pool of C3H mouse embryo cells was split and cultures were established in chemically defined medium NCTC 135 with either gelding horse serum (strain 5404) or fetal calf serum (strain 5405). Electron microscopy studies were made to determine presence of leukemia virus. Complement fixation tests were also carried out to determine presence of leukemia virus. Continuous morphological analysis with photography on the two strains has been made as well as on the subline from fetal calf serum switched to horse serum (strain 5778). Histochemical tests were the same as employed on previous study, NCI Serial No. 3212, project 3(b).

Major Findings: The cells in this study do not by electron microscopy or by complement fixation show the presence of leukemia virus. One line switched to horse serum after 407 days in fetal calf serum has produced tumors. This is an extremely important detail which has been of concern to us because the strain has been on fetal calf serum for so prolonged a period (407 days) and some reservation existed that it could be converted neoplastically with horse serum. The cells in the fetal calf serum are still not producing tumors. Morphologic and cytologic characteristics of neoplasia were observed in the cultures of a sister line in horse serum, from explantation time, at the time the horse serum line was producing tumors, whereas the sister line in fetal calf serum from the same pool did not show these changes. The morphologic criteria of neoplasia were a progressive increase in cytoplasmic basophilia, increase in nuclear cytoplasmic ratio, nucleolar size, and number. These are commonly used in clinical diagnosis.

Significance: There appear to be, at least in these initial studies, morphologic, cytologic, and cytochemical criteria that can be applied to distinguish the differences between cells grown from a common pool in either horse serum or fetal calf serum. These criteria appear to be best applicable when used in comparing the same pool of cells, one line in fetal calf serum and not producing tumors and the other in horse serum and producing tumors. The progressive changes, increases in basophilia, increased nucleo-cytoplasmic ratio, etc., resulting in the final criteria seem to slightly precede the actual demonstration of the neoplastic transformation. Further significance of the criteria may become apparent as data on the line on fetal calf serum switched to horse

serum are obtained and again correlated with the neoplastic transformation evidenced by tumor production. It is extremely significant to learn that a strain of cells carried for 407 days in fetal calf serum can still undergo neoplastic conversion in horse serum. This is possibly the longest a strain has been in fetal calf serum and still when switched to horse serum underwent conversion. We had been concerned that the fetal calf serum might so completely select out non-transformable cells or irreversibly stabilize the cells so that the horse serum would prove not to be effective as a conversion agent.

Proposed Course of Work: This study projected last year as possibly to be completed in a few months now has become exceptionally interesting and a fertile source of information. Correlations will be made if possible between the 3 lines of cells in the 2 media with particular emphasis on uncovering differences in the cells on horse serum and fetal calf serum and those on fetal calf serum switched to horse serum. An effort will be made to see if the changes seen in the strain switched to horse serum (strain 5778) are similar to those changes seen (strain 5404) in horse serum from explantation until the tumors from each are obtained. The gradual changes in 5778 from its parent 5405 will be noted for possible relation to induction of neoplasia. The tumor from 5778 cells mentioned in major findings and significance will be examined, cultured, and followed histochemically and for chromosomal complement to try to relate cells from tumors with cultured cells and to shed light on the possible significance of cell selection. All findings will in turn be compared with the findings in the study preceding this one on strain 4823 and its sublines. An effort will be made to determine if there are any common likenesses between the two different pools of cells from the inbred C3H mouse cells as they become neoplastic in vitro. If such proves to be the case, an endeavor will be made to try to determine or at least to postulate the mechanisms responsible for these results for additional analysis. Further, an analysis will be made to see if the results suggest any parameters other than production of tumors can be found to indicate incontrovertibly that cells are becoming or have become neoplastic.

Project Description (d): Isolation of leukemia virus from a strain of cells isolated in chemically defined medium for determination of ability to neoplastically transform cells in vitro.

Objective: To determine if leukemia virus in cells in vitro can be a causative agent in the "spontaneous" neoplastic transformation in vitro.

Methods Employed: Large cultures of strain 4705, a cell strain isolated and cultured in vitro, were tested by Dr. Janet Hartley by complement fixation antigen tests and electron microscopy. Tumors from these cells were similarly tested. Virus was recovered by Moloney isolation procedure from both tumors and the cells and fluid in vitro. Cells of non-neoplastic virus-free strain 4823 were infected with the virus, as well as Swiss mouse embryo cells. Complement fixing antigen tests and electron microscopy examination were used to determine infectivity of the non-neoplastic leukemia virus-free strain 4823. Intramuscular injections and eye implants of cells were made into AlbC and C3H/HeN mice.

Major Findings: From 40 discrete mouse tissue culture cell lines examined by electron microscopy or complement fixation, or both, for the presence of detectable virus, one (NCTC 4705), initiated and maintained on chemically defined medium, was chosen for a more extensive study. Virus-like particles (100 to 110 mu), morphologically similar to previously reported immature and mature C-type leukemia virus particles, were found budding from the plasma membrane and free in the intracellular spaces of cells in tissue culture and in fibrosarcomas resulting from intramuscular implants of these tissue cultures. Complement-fixation tests for group reactive murine leukemia antigens were positive, with titers consistently higher to a broadly reactive anti-serum than to anti-Friend, anti-Moloney, or anti-Rauscher sera. The 4705 virus was neutralized by Gross antiserum, but not by the F-M-R antisera. When injected into DD, BALB/c, or C3H/He newborn mice, the virus thus far has manifested no leukemogenicity, though virus from tumor extracts and tissue culture medium has been shown to be capable of infecting C3H and Swiss mouse embryo tissue cultures and successfully replicating in them. The role of the virus in accelerating or inducing neoplastic transformation in NCTC 4705 is still not known. When it was introduced into NCTC 4823, a non-neoplastic cell line in other respects similar to NCTC 4705, 4823 manifested no signs of neoplastic transformation after harboring the virus more than 300 days in vitro. A comprehensive electron microscope study has been made of the morphology of the cells and virus from 4705 cells in both the 4705 cells and tumors from them, both Swiss cells in culture and 4823 cells exposed to the virus isolated from the 4708 tumors. Also pellets of the used medium from 4705 cells Swiss embryo cells, were examined. All showed the presence of typical type C particles like those in the original 4705 cells. A manuscript has been published on the entire study.

Significance: Clearly the electron microscope study and the CF tests demonstrate that the leukemia virus isolated from strain 4705 and the tumors from the 4705 cells infect strain 4823. Nevertheless, the data from implantations to date show that the infected cells have not become neoplastically transformed. The findings here further confirm the information that the neoplastic transformation occurs in vitro in cells both with and without leukemia virus. Unless the strain of animal used for the tumor test is unsatisfactory (and it was the strain from which the original virus was isolated), the leukemia virus does not appear to account for the "spontaneous" neoplastic transformation obtained in cells from numerous studies of C3H mice. The inability of a leukemia-like particle to induce leukemia does not disprove the infective capacity of that particle. The 4705 virus provides an example of such a case. Although morphologically similar to a leukemia virus, antigenically similar to a leukemia virus, and neutralized by antiserum to a leukemia virus, it does not cause leukemia when injected into newborn mice. It can, however, infect virus-free tissue cultures and successfully replicate in them.

Proposed Course of Work: This particular study has culminated, other than waiting for long-term holding of Swiss mice to determine if any tumors will arise in Swiss mice.

Serial No. NCI-3213, Project 1 (Same)
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Carcinogenesis in vitro.

Principal Investigator: K. K. Sanford

Other Investigators: R. Parshad, J. L. Jackson, R. R. Gantt, J. T. Mitchell, B. C. Smith, H. A. Kerr, and C. U. Garrison.

Cooperating Units: Dr. T. Dunn, Laboratory of Pathology, NCI.

Project Description:

Objectives: 1. To elucidate the cause(s) of "spontaneous" neoplastic transformation of mammalian tissue cells in vitro.
2. To develop methods for inducing neoplastic transformation that will be rapid, controlled, and reproducible for both short term strains and established non-neoplastic cell lines.
3. To obtain clones of stable, non-neoplastic cells that can be converted to neoplastic cells by methods developed in #2 above and thus provide quantities of neoplastic and non-neoplastic cells from one cell for biochemical studies. The use of clones developed from one cell is essential for controlled biologic material and for eliminating the possibility that the carcinogenic agent is selecting certain cell types rather than inducing neoplastic change.

Methods Employed: Experiments were designed to examine the following factors that may influence or cause neoplastic transformation of cells in vitro: Type of serum or serum fractions as supplements to chemically defined medium, oxygen tension, chemical carcinogens (4-nitroquinoline-oxide and methyl-nitrosoures). All tests for neoplastic transformation were made by implanting cells into animals of the strain from which the cells originated.

A large group of cloning methods by platings and single cell isolations were tested and new methods were tried in numerous efforts to obtain clones that we could be assured originated from only one cell. These efforts are still in progress.

Major Findings: Effect of type of serum and absence of serum on neoplastic conversion in vitro.

1. Previous observations from this laboratory (J Nat Cancer Inst 36:953-963, 1966) that neoplastic conversions of C3H/HeN and C3H$_f$/HeN mouse embryo cells occur reproducibly after approximately 90-180 days of culture in NCTC 135 +

10% horse serum have been confirmed. Data on 9 cell lines from 6 different pools of cells support this conclusion.

2. The previous observation (J Nat Cancer Inst 37:247-249, 1966) that fetal calf serum prevents or at least delays this conversion has been confirmed. Data on 5 cell lines from 4 different pools of $C3H_f$ mouse embryo cells support this conclusion.

3. Calf serum, as distinct from fetal calf serum, acts like horse serum in bringing about early neoplastic conversion of $C3H_f$ mouse embryo cell lines. Data on 4 cell lines from 3 different embryo pools support this conclusion.

4. Fetal calf serum added to horse serum or calf serum may prevent the neoplastic conversion that occurred in horse serum or calf serum-supplemented medium alone. Data on two cell lines from one pool of embryo cells support this conclusion. Thus fetal calf serum has been shown to play an active role in preventing neoplastic conversion of mouse embryo cells in vitro.

5. Fetuin, the major protein component of fetal calf serum, when prepared by one ammonium sulfate precipitation from fetal calf serum and added to calf serum has delayed neoplastic conversion in one line and appears to have reduced tumorigenic capacity of cells in a second line. However, further purification of fetuin by a second precipitation has failed to prevent neoplastic conversion when fetuin was added to horse serum. In fact, conversion occurred earlier than in horse serum alone.

6. Results to date suggest that increasing the concentration of horse serum to 40% may also shorten the time for neoplastic conversion as compared with cells grown in medium with 10 or 20% horse serum.

7. Efforts to convert cell lines by transfer to horse serum after relatively short periods in fetal calf serum have been successful in at least one cell line. However, as the period of culture in fetal calf serum is prolonged to several years, such transfers have not been successful in inducing conversion in three of the cell lines tested from two different cell pools. These observations suggest that the fetal calf serum action may be one of selecting from the cell population cell types that are more stable with respect to neoplastic conversion.

8. Embryo cells from the germ-free ALB-2T mouse have been shown in 6 lines from 2 different pools to undergo reproducible neoplastic conversion after about 150 days in vitro when grown in NCTC 135 + 10% horse serum. These findings prove that the serum induced neoplastic conversion applies to several strains of mice.

9. In 5 additional lines from ALB-2T embryo cells of two different pools the cells in fetal calf serum medium have failed to undergo neoplastic conversion, have showed a delayed response or lowered incidence of tumors. Thus, the same type of serum effect observed with C3H cells has been demonstrated on cells from a different mouse strain.

10. Embryo cells of one line from the strain A mouse have undergone neoplastic conversion, whereas cells from the same pool in fetal calf serum still test as non-neoplastic. These observations prove that the fetal calf serum effect applies to cells from several strains of mice.

11. Embryo cells from the Albany rat grown in NCTC 135 + horse serum from 3 different cell pools all underwent neoplastic conversion but after much more prolonged culture in vitro than any of the mouse cells. Lines in fetal calf serum have not as yet undergone neoplastic conversion, but longer periods are necessary to evaluate the relative effects of these two sera on the rat cells.

12. From a limited number of assays of rat embryo cells initiated and carried for almost 3 years in protein-free chemically defined medium, these cells appear to be non-neoplastic.

Effect of low and high oxygen level on neoplastic conversion of $C3H_f$ mouse embryo cells grown in NCTC 135 + 10% horse serum.

1. $C3H_f$ mouse embryo cells grown under 60% or 40% O_2 administered by a one-second jet/minute failed to grow and ultimately cultures declined in cell numbers, whereas under 20%, 2%, or 1% O_2 rapid cell proliferation was obtained.

2. Cells grown under 1% O_2 became neoplastic at approximately the same time as cells grown at 20% CO_2. Thus low O_2 tension under conditions of these experiments failed to accelerate neoplastic conversion and high O_2 tension was inhibitory to cell survival and continued growth.

Effect of the chemical carcinogens 4-nitroquinoline oxide and methylnitrosourea on mouse and hamster cells in vitro. (See also project of Dr. J. T. Mitchell, NCI-3233.)

$C3H_f$ mouse embryo cells and Syrian hamster embryo cells have been treated with the two chemical carcinogens, have been prepared for cytologic and chromosome study, and are currently under assay for neoplastic conversion.

Cloning of mouse, hamster, and rat embryo cells in vitro.

1. Single, isolated cells from cultures of non-neoplastic lines of mouse, rat, and hamster cells grown in fetal calf serum medium are exceedingly difficult to grow. To date, after many efforts, lines from 2 or 3 rat cells have been established. None from one cell.

2. Plating and other feeder-layer methods used by many workers have been evaluated and found inadequate to meet our requirements for absolute certainty that the cells arise from one cell.

3. Several new methods are being developed and one of these is currently under test.

Significance:

Effect of type of serum and absence of serum on neoplastic conversion in vitro.

Certain of these observations are particularly significant in developing concepts concerning the cause of "spontaneous" neoplastic conversion in vitro.

First, we have extended the earlier observations on reproducibility of neoplastic response in the C3H mouse cells to embryo cells of the germ-free ALB-2T, $C3H_f$, and strain A mouse, the Albany rat, and the Syrian hamster. (J Nat Cancer Inst 39:691-703, 1967). This conversion in almost all cell lines studied of the mouse is delayed or prevented by replacing the horse serum supplement to NCTC 135 with fetal calf serum. Further, several of these cell lines have been examined for 11 murine viruses and mouse leukemia virus and found to be negative by current tests. We conclude, therefore, that some factor in serum is playing a major role in carcinogenesis. Two hypotheses were presented earlier (J Nat Cancer Inst 37:247-249, 1966). 1) that a component(s) of horse serum is carcinogenic, or 2) that a component(s) of fetal calf serum acts as a repressor preventing neoplastic conversion. Finding #4 indicates that fetal calf serum is active and justifies further intensive analysis of its components. Finding #3 indicates that calf serum does not act like fetal calf serum in preventing neoplastic conversion and may be substituted for gelding horse serum in bringing about early neoplastic conversion. Thus a serum more closely related to fetal calf serum has been pinpointed for further analysis. The observation is important also in eliminating certain hypotheses based on peculiarities of gelding horse serum. Finding #6 suggests that the first hypothesis may also be true and that horse serum may be carcinogenic.

Finding #11 is significant in providing more suitable stable material for carcinogenic studies in vitro. In addition, the rat chromosomes are far more suitable for analyzing cytogenetic effects than mouse chromosomes. The establishment of stable cell lines for use with carcinogenic agents such as tumor viruses or chemical carcinogens is an important step in obtaining good biological material for further experimental studies in carcinogenesis.

Finding #7 is significant in suggesting that the serum effect may be one of selecting cell types and is sufficiently important to warrant intensive efforts to clone cells in order to test this hypothesis. It is important to know whether the mechanism of the serum action is one of cell selection or induction of cell change.

Effect of low and high oxygen levels on neoplastic conversion of $C3H_f$ mouse embryo cells grown in NCTC 135 + 10% horse serum.

In a previous study of ours (J Nat Cancer Inst 35:719-725, 1965) testing the effect of intermittent anaerobiosis on neoplastic conversion, two cell lines that had been carried in culture for some time were exposed to intermittent anaerobiosis. Both control unexposed and experimental cultures underwent neoplastic conversion at the same time. An objection to this study was raised

that the prior growth of the cells in mass culture might have resulted in anaerobiosis that triggered the neoplastic conversion observed later in both treated and control lines. Therefore, we initiated cells in culture under continuous gassing at high and low O_2 levels. Still, no significant differences appear between cell lines carried at 20% or 1% O_2. These data strongly suggest that the neoplastic conversion in these cells is not related to low O_2 tension. The higher O_2 tensions were also found to be inhibitory to cell survival and growth.

Effect of chemical carcinogens on mouse and hamster cells in vitro.

With the development of more stable cell lines in fetal calf serum, it is now essential to know that these can be rapidly converted by chemical carcinogens or tumor viruses in order to fulfill our objective #3.

Cloning of cells in vitro.

Cultures initiated from any tissue usually contain many different cell types. In order to detect subtle biochemical differences associated with neoplastic conversion, more homogeneous biological material is needed. Also, a clone is necessary in order to establish whether the mechanism of the serum action on neoplastic conversion is induction or cell selection.

Proposed Course of Study: Further intensive efforts at cloning the non-neoplastic cell lines will be continued. Both chemical carcinogens and polyoma virus will be used as carcinogenic agents in efforts to attain objective 3. At this stage our efforts will then be directly associated with the biochemistry program of Drs. Gantt and Hardy on materials we make available to them. Many of these may have to be adapted to micro-carrier type suspension cultures. We are currently exploring this type of culture. Manuscripts will be prepared on the effects of fetal calf serum and fetuin in preventing neoplastic conversion and on the effects of low and high O_2 tensions on neoplastic conversion.

Honors, Awards, and Other Activities:

Member, Advisory Committee of the Animal Cell Culture Collection of the American Type Culture Collection.

Invitation to speak at the symposium on "Normal and Malignant Cell Growth" at the University of Chicago.

Publications:

Hall, W.T., Andresen, W.F., Sanford, K.K., Evans, V.J., and Hartley, J.W.: Virus particles and murine leukemia virus complement-fixing antigen in neoplastic and nonneoplastic cell lines. Science 156: 85-88, 1967.

Sanford, K.K.: "Spontaneous" neoplastic transformation of cells in vitro: Some facts and theories. Nat Cancer Inst Monogr 26: 387-418, 1967.

Sanford, K.K. and Hoemann, R.E.: Neoplastic transformation of mouse and hamster cells in vitro with and without polyoma virus. J Nat Cancer Inst 39: 691-703, 1967.

Publications (continued):

Hall, W.T., Hartley, J.W., and Sanford, K.K.: Characteristics of and relationship between C-particles and intracisternal A-particles in cloned cell strains. J Virology 2: 238-247, 1968.

Hall, W.T., and Sanford, K.K.: Virus particles in eight murine cell strains of single cell origin. J Cell Biol 34: 52-53A, 1967.

NCI Serial NO. 3213, Project 2 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Search for "indicators" of neoplastic conversion in vitro.

Principal Investigator: K. K. Sanford

Other Investigators: R. Parshad, J.L. Jackson, B.B. Westfall, B.C.Smith, C.U. Garrison, G.L. Carter, and E.V. Peppers.

Cooperating Units: B.E. Barker, Department of Pathology, Rhode Island Hospital, Providence, R.I.; M.W. Woods, Laboratory of Biochemistry,NCI; D.B.McN. Scott, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa.; H. Rapp, Biology Branch,NCI.

Project Description:

Objective: To identify and characterize properties of cells in culture directly associated with their normal or neoplastic state in an effort to analyze the nature of the neoplastic change.

Methods Employed: Cells during various stages of in vitro growth and neoplastic conversion were examined for: 1) Cytologic changes, 2) Cytogenetic changes, 3) Changes in proliferation rates, 4) Changes in glucose metabolism, and 5) Changes in Forssman antigen. Cells were studied in Wright-Giemsa stained coverslip preparations, and by standard chromosome methods. Aliquots of cell suspensions were also assayed for neoplastic or non-neoplastic state by intraocular or intramuscular implantation. Glucose uptake and lactic acid production were determined by chemical analyses on used culture fluids. Growth rates in vitro were estimated by enumeration of cell nuclei in replicate cultures. Manometric measurements of anaerobic glycolysis and enzyme activity measurements were carried out on counted cell suspensions.

Major Findings:

Cytologic changes during neoplastic conversion of mouse, hamster, and rat embryo cells in vitro.

1. A fairly good correlation appeared between cytologic criteria and demonstrated neoplastic state.

2. Certain common patterns of change in the different cell lines during in vitro neoplastic conversion have been noted. These are being analyzed further for their value as "indicators" of neoplastic conversion. Photographic documentation is now completed and the manuscript will be prepared soon for publication.

Cytogenetic changes during neoplastic conversion in vitro.

1. Calf and bovine sera, like fetal calf serum, reduced the rate of

heteroploidization and appearance of abnormal chromosomes as compared with horse serum in cultured cells from $C3H_f$ mouse embryos. Thus, the calf and bovine sera seem to contain some factor(s) common to fetal calf serum or lack the factor(s) in horse serum that influences chromosomal stability.

2. From the distribution and morphology of the abnormal chromosomes in the mouse embryo cells we conclude that the chromosome breaks during in vitro growth in all sera tested are localized at or near the centromere.

A manuscript has been submitted for publication entitled "Effects of horse serum, fetal calf serum, calf serum, bovine serum, and fetuin on neoplastic conversion and chromosomes of mouse embryo cells in vitro."

3. Chromosomes in embryonic cells of the germ-free ALB-2T mouse showed greater stability in number and fewer abnormalities during long-term culture than those of the $C3H_f$/HeN mouse.

4. Cells in the diploid range predominantly contributed to the growth of the tumor when tissue culture cells were implanted into mice.

5. Cells of the tumors showed certain of the marker chromosomes and abnormalities observed in vitro.

Data from these studies are still being analyzed.

6. Chromosomes of the rat embryo cells during long-term culture appear to be more stable with respect to changes in number and morphology than those of the mouse.

Data are still being collected and analyzed to see if any common pattern of chromosome change can be associated with onset of neoplastic conversion in the rat chromosomes.

Changes in growth rates and glucose metabolism.

A. Stability in vitro of diverse capacities for tumor-production and glucose metabolism in three murine clones derived from the same cell.

1. Clones maintained for about 8 years in NCTC 135 + 10% horse serum have retained their characteristic differences in tumor-producing capacity from high line clone with high incidence of tumors and short latent periods to intermediate and low line clones with progressively lower percentages of tumors and longer latent periods.

2. When implanted in x-irradiated hosts the clones maintained their characteristic differences in tumor latent periods.

3. The clones retained their characteristic glycolytic properties which tended to correlate with their in vivo growth characteristics; that is, the high line clone had the highest rate of anaerobic glycolysis. Intermediate and low line clones also differed in glycolytic rate. The rate of glycolysis appeared to be unaffected by numbers of cells per vessel.

4. Under aerobic conditions growth rates of the three clones were similar.

5. Aerobic glycolytic rates tended to vary inversely with inoculum size, and differences between clones during aerobic growth were not conspicuous.

6. Under anaerobic growth conditions, cells showed a marked "Pasteur effect" and slight differences between them in total glucose uptake and lactic acid accumulation in the medium.

7. Although data at first suggested a better survival of high line cells under anaerobic conditions, experiments with cinematographic monitoring failed to confirm this. In the absence of O_2, cells of all clones appeared to survive but mitosis was prevented.

8. Cells of all three clones were successfully adapted to continuous growth in chemically defined, protein-free medium.

9. In the chemically defined medium, cells of all three clones could grow as tumors from smaller numbers of cells than in serum-containing medium.

10. The low line clone in chemically defined medium showed increased tumorigenic capacity comparable to that of the high line clone even though glycolytic rate differences persisted.

11. Examination of enzymes of the glucose monophosphate shunt (G6PD, PGD, and hexokinase) have been measured for comparison with the earlier values. Data on the enzyme activities are currently being analyzed. A manuscript on this study will be prepared for publication.

B. Growth, glycolysis, and neoplastic conversion in vitro of embryo cells from the germ-free ALB-2T mouse.

From a pool of embryo cells a line was initiated on horse serum medium and one on fetal calf serum medium. These lines were examined for neoplastic conversion, cytologic changes, chromosomal alterations, rates of cell proliferation, levels of the stationary phase of growth, glucose uptake, lactic acid accumulation, and anaerobic glycolysis. Data from this study are currently being analyzed to see if any of these properties especially those relating to growth and glucose metabolism can be directly associated with neoplastic conversion.

Changes in Forssman antigen.

1. Syrian hamster embryo cells contain Forssman antigen which persists for some time during their culture in vitro.

2. Forssman antigen tends to disappear from the cells during prolonged culture and the presence or absence of antigen appears unrelated to neoplastic or non-neoplastic state of the cells.

Significance:

Probably the most distinguishing feature of neoplastic or malignant cells is their capacity for uncontrolled, invasive growth in the organism. For this reason, in studying the neoplastic transformation of cells in vitro, we have tested their capacity to grow as malignant neoplasms when implanted in animals of the strain of origin. This type of assay is slow, calls for large numbers of cells, is usually limited to cells from inbred animals, and is difficult to quantitate. It would be desirable to be able to recognize neoplastic cells directly in culture. Any property of cells in vitro that could serve as a diagnostic criterion of neoplastic state might also provide clues to the nature of the neoplastic changes.

Several responses of mammalian tissue cells to long-term culture in horse or fetal calf serum-supplemented medium have been studied in the hope of identifying and characterizing properties directly associated with the normal or neoplastic state of the cells.

Cytologic changes: Certain of the cytologic changes may provide sensitive "indicators" providing means of diagnosing the neoplastic or non-neoplastic state of the culture or of individual cells.

Chromosomal alterations: The fact that fetal calf serum as compared with horse serum appeared to stabilize the chromosomes and also delay or prevent neoplastic conversion suggested some correlation between chromosome stability and maintenance of non-neoplastic state. A significant observation is that such chromosomal stability also occurs with calf serum supplement which does not delay or prevent neoplastic conversion. This observation thus fails to support the first concept.

With respect to the mouse chromosomes, there appears to be no pattern of change in numbers or structure that can be rigidly correlated with neoplastic conversion. In rat or Chinese hamster cells, chromosomes can be better identified and possibly some such pattern may emerge.

Changes in proliferation rates: It is important to know whether a neoplastic cell population differs in growth potential in vitro from a non-neoplastic population. Such differences, if present, could provide clues as to basic nutritional or biochemical differences. To date, no conspicuous differences appear.

Changes in glucose metabolism: In our studies we have been trying to establish firmly whether neoplastic cells in vitro differ in glucose metabolism from the populations of non-neoplastic cells from which they arise.

Changes in Forssman antigen: The Forssman antigen content of carcinogen-induced guinea pig hepatomas (J Nat Cancer Inst 36:529-538, 1966) and of certain mouse tumors (J Immunol 77:305, 1956) has been reported as higher than in normal tissues. For this reason, it seemed of interest to know whether a higher concentration developed during neoplastic conversion of cells in vitro.

Proposed Course: These studies will be completed for publication.

Publications:

Parshad, R., and Sanford, K. K.: Effect of horse serum and fetal calf serum on stability of chromosomes in mouse embryo cells *in vitro*. J Nat Cancer Inst 39: 143-152, 1967.

Sanford, K. K., Barker, B. E., Woods, M. W., Parshad, R., and Law, L. W.: Search for "indicators" of neoplastic conversion *in vitro*. J Nat Cancer Inst 39: 705-733, 1967.

NCI Serial No. 3214 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of cell metabolism *in vitro*.

Principal Investigator: Benton B. Westfall

Other Investigators: E. V. Peppers, V. J. Evans, K. K. Sanford, J. C. Bryant, H. A. Kerr, F. M. Price, G. L. Carter, R. N. Altland, J. L. Jackson, B. C. Smith, and J. R. Martin

Man Years: (See Serial No. NCI-3211)

Project Description:

This project is part of the continuing study concerned with the biologic origin and character of cancer cells *in vitro* and *in vivo*. The general course of the studies has not materially changed since the 1967 report. The project is mainly concerned with numerous biochemical approaches and application of such techniques to the study of cells and their supporting media. It has several parts as noted below under "Findings".

Objective: As in 1967, the continuing objective is to learn more about the cancerous process in cells with emphasis on : 1) Molecular changes that may take place in the cells as they become able to produce tumors on injection into animals after cultivation *in vitro*; 2) behavior of cells from various species and of different tissue origin; 3) cells of tumor-producing potential, but differing in this potential; 4) cells grown under several conditions including type of vessel, kind of medium, medium supplements, and the general chemical and physical environment of the cells.

Methods Employed: Cells and their culture media from the on-going program of the Tissue Culture Section were used, so that 1) all background information on history, culture, type of medium treatment of cells, preparation and handling of the media were immediately and fully available; and 2) no storage or transportation problems of the cells or media were involved. Continuing adaptation, modification, invention, and validation of methods for the study of the cells under a wide range of carefully controlled conditions were used. Classical methods have been used where possible, new ones were devised when necessary, and old ones adapted for use for the specific demands of cells in culture. This adaptation was frequently needed because of the limited amount of tissue from the early pre-neoplastic stages of cultured cells.

Major Findings: The paper "Taxonomic Enzymology" noted in last year's report as given orally before the Second Decennial Review Conference last

year, has now been published in NCI Monograph #26, as noted below. These studies include analyses of the relative activities of certain enzymes of the cells, namely arginase, acid phosphatase, lactate dehydrogenase, alkaline phosphatase, and malate dehydrogenase. These enzyme activities were determined on cell lines grown continuously in chemically defined media (NCTC 109, 117, and 135) containing no added protein supplement. Cell strains of mouse, hamster, monkey, and human origin were included. Lactate and malate dehydrogenase activities persisted at a very high level. Some of the other enzymes, namely arginase and alkaline phosphatase, were found to vary considerably with conditions of cell culture. For example, arginase activity decreased in mouse cells on chemically defined medium as compared with the same cell strain on protein-rich serum-supplemented medium.

Another major finding with cells in vitro is that the liver cells continue to have a high arginase activity; in fact, there appears to be almost as much arginase activity in them as in C3H mouse liver cells in vivo. The production of urea, however, is negligible, suggesting loss of a critical liver function. The usual function of arginase is to convert arginine to urea and ornithine but in these cells it does not appear to do so. Thus it is not here a mediator in the Krebs cycle. It appears in these cells to function in making protein and not as an enzyme source. Studies on these strains have been continued.

The catalase activity is low in the liver cells in comparison with whole liver, but high in comparison with the strain L fibroblasts; it is also low in the liver cells though the arginase activity is high. This indicates that enzyme activities in cells can change independently of other enzyme activities in the same cells. It is reputed that rodent cells in culture lose practically all alkaline phosphatase activity. However, we have found that of all cell strains studied from man, monkey, hamster, or mouse, a rodent strain, Strain L-1210, growing in roller tube fluid suspension cultures in serum-supplemented medium, had the highest alkaline phosphatase activity. When this cell strain was adapted to growth in chemically defined medium by gradual depletion of serum to only a fraction of one percent serum v/v the alkaline phosphatase activity decreased. Nevertheless, this alkaline phosphatase activity was a feature distinguishing these cells from fibroblast-like cells. Four human strains studied, two from tumors and two from normal tissues, were found to have measurable arginase activity. Cells adapted to growth in chemically defined medium show less protein production, indicating unneeded protein may not be made. Studies on these strains have been continued.

In addition to determinations of enzyme activities in cells, other biochemical markers in cells have been identified and measurements of DNA and RNA of the cells have been made. Both human skin epithelium in vitro and C3H mouse liver cells (strain 721) in vitro showed high storage of glycogen, distinguishing them from many other cell strains from man, monkey, hamster, or mouse. These two strains can be distinguished from each other by their distinctive differences in arginase activity. The liver cells have at least twice the arginase activity that the skin cells have. Studies on these strains have been continued.

A study of the "Sialic Acid" content of the various cell strains with respect to the total protein content of the cells has been undertaken. Also,

this parameter has been examined in the protein-containing media containing either horse serum, chick embryo extract, or fetal calf serum. Fetal calf serum has a higher sialic acid content than horse serum. The results are being analyzed.

The data on the activities of arginase, alkaline phosphatase, and acid phosphatase show no significant differences in cells in either cell strain in fetal calf serum, with one exception. Strain 4823 in fetal calf serum is normal for arginase activity; even this value is regarded as minimal if the strain were to be assayed more intensively. The other cells appear to have lost most of their arginase activity. Similarly, acid phosphatase activity has also been decreased with one exception, which was also in cells carried in fetal calf serum. No correlations can be made between the changes in the three enzyme activities and the corresponding changes from normal cells to neoplastic cells. At high activity levels product inhibition and an insufficient cell mass in some cases prevented repetition of experiments with lower amounts of the enzyme. Other studies on glycolytic activities of high and low tumor-producing clones are discussed in Serial No. NCI-3213, project 2. Studies on these strains have been continued.

Manuscripts are being prepared under the tentative titles listed below:

1. Persistence of high arginase activity in murine liver during 20 years in cell culture.
2. Dry weight, protein and DNA content of murine cells grown for long-term in chemically characterized medium.
3. High alkaline phosphatase activity of certain malignant murine cell strains in long-term culture.
4. Lack of great variability in acid phosphatase activity of 20 murine cell strains in long-term culture.
5. Lactate dehydrogenase activity of 20 long-term murine tissue cell strains.
6. Arginase, acid phosphatase, alkaline phosphatase, malate dehydrogenase and lactate dehydrogenase activity of kidney cells derived from Green and Rhesus Monkey growing in chemically characterized medium.

Significance: The study has been predicated on the following hypotheses: 1) enzymes are the mediators of the exquisitely detailed metabolism of all living organisms; 2) enzyme action depends on precise genetically determined structure; 3) the fitness of a chemical substance for its biological function is not changed by growth in vitro or by the tumor process. The cells adapted to growth in completely protein-free media are particularly valuable for enzyme studies because serum itself contains enzymes that become complicating factors.

Additional evidence has been gathered giving support to our previously reported concept, that hereditary differences in enzyme activity and tumor-producing capacity tend to develop during long-term cultivation in vitro of mammalian cells. Biochemical markers appear to be increasingly useful in identifying and evaluating these differences. Such markers should be useful in current research on the neoplastic change for characterizing the cells before, during, and after the neoplastic transformation.

Proposed Course of Project: Studies will be continued along the course described herein. The currently studied enzyme systems will be further investigated. More cell strains are being studied as additional strains become adapted to chemically characterized medium and as strains of pre-neoplastic and post-neoplastic cells become available for comparative study; the latter strains in particular are of increasing importance. The adaptation, modification, and invention of methods applicable to the project are being continued.

Publications:

Westfall, Benton B.: The taxonomic enzymology of tissue culture cells. Proceedings of Second Decennial Review Conference, Bedford Springs, Pennsylvania, September 11-15, 1966. Published in NCI Monograph #26: 229-246, 1967.

NCI Serial No. 3215 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies on mammalian cells in agitated fluid suspension cultures and in large stationary cultures.

Principal Investigators: J. C. Bryant and V. J. Evans

Other Investigators: H. A. Kerr and F. M. Price

Man Years: See NCI-3211

Project Description:

Objectives: 1) To define factors governing the growth of established mammalian cells in large agitated fluid suspension cultures and large stationary cultures, particularly with respect to rates of cell proliferation and rates of glucose utilization.

2) To grow large numbers of cells in both kinds of large cultures within relatively short periods of time, as desired, for storage in liquid nitrogen, and for initiating larger continuous flow cultures (NCI Serial No. 3220).

3) To adapt additional strains of cells, already established in stationary tissue cultures, to growth in agitated fluid suspension cultures, particularly non-neoplastic strains of mouse embryo cells.

Methods Employed:

1) Shaker flasks (described in previous reports) of 500-ml, 250-ml, or 125-ml size, with relatively short necks can be centrifuged directly at fluid change. The flasks are on a gyrotary shaker platform oscillating at about 90-100 rpm. Each fluid change is complete; small samples of cell suspension for cell counts and samples of used supernatant fluid for reducing sugar analyses provide reliable monitoring of each culture without sacrificing the culture. Continuous aeration with humidified 10% CO_2 in air was provided through two cotton traps in the flask stopper by means of a multiple solenoid valve gas-metering device. Cultures in all experiments received chemically defined medium NCTC 135 supplemented with methylcellulose.

2) A standard slowly rotating drum apparatus for roller tube cultures was fitted with a special speed controller and electric motor and reducing gears so to deliver a wide range of relatively "mild" higher speeds, about 10 to 60 rpm. Standard test tubes (16-18 mm diameter) were used as culture flasks instead of our regular roller tubes (25 mm diameter). Cells were from established mouse embryo strains.

3) Large stationary "Povitsky" bottles of about 2 liters gross volume were used in essentially the same way as the shaker flasks, except that it was possible to take samples only for reducing sugar determinations, but not for cell counts, at each fluid change. Also methylcellulose supplement was used only for part of the cultures.

Major Findings:

All experiments during the past year were designed to bring about growth and proliferation of non-neoplastic cells in suspension cultures. Two different kinds of suspension cultures were used: shaker flasks and roller test tubes. In several experiments with shaker cultures the non-neoplastic cells in medium 135 plus 10% fetal calf serum disintegrated markedly within a few days, while cells of the matching neoplastic strain either proliferated or remained viable. In the non-neoplastic cell cultures the remaining cells that had not disintegrated nevertheless failed to grow when transferred to stationary T-15 flasks.

The milder type of fluid agitation provided by the roller culture flasks in the special multi-speed drum was then tried. In several exploratory experiments with cells of non-neoplastic strains, speeds of 37 and 50 rpm failed to yield any viable cells in suspension, although some cells did attach to the walls at the lower speed.

Work reported last year on some comparisons between large shaker flask cultures and large stationary cultures, with cells of strain 2071-L and two other strains in chemically defined medium, was written up as a manuscript for publication.

Significance:

The many attempts to grow cells of non-neoplastic strains in fluid suspension cultures have been uniformly unsuccessful, both in this laboratory and to our knowledge in other laboratories. In contrast, cells of most established neoplastic strains usually proliferate readily in suspension. We propose, as a working hypothesis, that the non-neoplastic cells might be induced to grow in roller tubes at some intermediate speed of rotation, where the kind of fluid agitation would be relatively gentle and yet sufficient to keep the cells in suspension instead of attached to the flask surface. Such intermediate conditions of fluid agitation in roller tubes have been found previously (J.C. Bryant, PhD thesis, Georgetown University 1963) to allow growth of a fragile strain of monkey kidney cells in roller tube cultures; the chemically defined medium was wholly unsupplemented even with methylcellulose, while cells of the same strain usually would not proliferate in the same medium with methylcellulose, when in shaker flask cultures.

The advantages of suspension cultures in flasks that can be centrifuged directly at fluid change have been described in previous reports. Small samples of cell suspension for cell counts can be withdrawn at each fluid change or more often if desired. Continuous aeration insures close control of the supply of oxygen uniformly available to all of the suspended cells.

The medium can be chemically defined if supplemented with methylcellulose, at least for many established neoplastic strains of cells. Thus the agitated fluid suspension culture can be used for experiments where immunological and serological complications resulting from the presence of serum are to be avoided.

Proposed Course of Study: Studies on the effect of environmental factors in suspension cultures of various kinds are being continued. The small roller tube variable speed apparatus will be used in further studies with non-neoplastic cells in order to search for a "breaking speed" that will prevent cell adhesion to the glass and at the same time permit proliferation of cells in suspension. Many different rotation speeds will be tried.

Honors and Awards: (J. C. Bryant)

1) Serves as Panel Member of the Interagency Board of U.S. Civil Service Examiners for the Washington, D.C. area.

2) Served as Session Chairman at the Annual Meeting of the Society for Cryobiology, Washington, D.C., August 7, 1967.

3) Serves as Visiting Lecturer at the Tissue Bank, National Naval Medical Center, Bethesda, Maryland.

4) Invited to visit and advise for two days on tissue culture laboratory problems at the Hormel Institute of the University of Minnesota.

5) Invited to present a paper on "Mammalian cells in chemically defined media in suspension culture" at the Annual Meeting of the Association for Gnotobiotics, June 10-11, 1968 at Buffalo, N. Y.

Publications: (J. C. Bryant)

J. C. Bryant: Book review of National Cancer Institute Monograph 26, September 1967, "Second Decennial Review Conference on Cell Tissue and Organ Culture" (Bedford, Pennsylvania, September 11-15, 1966) for Cancer Research 28: 000-000, 1968.

NCI Serial No. 3220 (Same)

1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development of mass stirred fluid culture system with a continuous nutrient fluid renewal.

Principal Investigators: W. F. Andresen, J. C. Bryant, R. R. Gantt, K. K. Sanford, and V. J. Evans.

Other Investigators: F. M. Price, H. A. Kerr, B. B. Westfall, J. L. Jackson, and J. R. Martin.

Man Years: See Serial No. 3211.

Project Description:

Objectives: To develop further a more precise control of the environment in a mass suspension culture through the following improvements: a) never having to remove the culture from its 37.5°C temperature water bath environment throughout the period of cultivation; b) developing a method of renewal of medium by which the concentrations of components are kept in balance with the specific rate at which they are utilized, with no loss of cells. This would produce and maintain more perfectly controlled culture environments. The fast and feast cycle of a complete periodic fluid renewal schedule would be eliminated and better emulation of in vivo growth conditions in particular could be obtained. We plan to establish fresh tissue of mouse and/or rat cells in fluid suspension culture and to culture finite life diploid human cells in fluid suspension culture.

Methods Employed: As the original major designer and operator (Mr. W.F. Andresen) of this unique cell culture system returned to graduate school to complete his Ph.D. requirements, there has been less intensive activity in this project until just recently. A system with the necessary components has been devised and developed to allow continual renewal, with negligible loss of cells, of the culture medium in mass suspension cultures. The system has been adapted to use with a greatly modified New Brunswick 5-liter fermentor. A constant temperature is maintained in the culture vessel by a surrounding circulating water bath held at 37.5°C constant temperature. Provisions have been made for the periodic withdrawal of small representative samples of the stirred cell suspension. In essence what this system is trying to accomplish is to keep the cells in suspension but at the same time have the centrally positioned fluid withdrawal unit function in three ways: keep cells

suspended, serve as fluid withdrawal pipe, and act as an angle centrifuge to spin out the cells in the withdrawal fluid and throw them back into the main part of the culture vessel. We are also trying to reduce cell waste products which must influence successive generations; these waste products can modify cell metabolism. We hope to attain controlled-state uniform behavior.

Major Findings: The nature of the fast variable fluid removal system has been found to give precise, even, consistent fluid renewal and removal because the removal of cell-free exhausted fluid is dependent on the proper relationship between the force of gravity on the cells and the slow constant upward flow of the culture fluid. A variable pump which provides even, consistent fluid removal has now been developed. It pumps sterile medium into the culture and simultaneously pumps, at the same rate, the exhausted culture fluid through a large diameter withdrawal tube and then, cell free, into a collecting flask.

The growth of strain L cells was vastly improved by a continuous replaced atmosphere. Glucose utilization is rapid, indicating considerable metabolic activity. To compensate for this activity a higher concentration of glucose was used initially in the renewal medium (300 mg percent instead of the usual 100 mg percent). But it was found that the depletion rate of glucose at the 100 mg percent level is more representative of the other components of the medium and this level is used exclusively. The feasibility of partially enclosing the culture vessel in a simple aquarium type circulating water bath held at constant temperature 37.5°C has been demonstrated for our purposes. The first system produced excellent results and, with fixed tissue cells, accomplished many of the original objectives. Growth of strain L in chemically defined medium was encouraging. At first, strain L cells growing in medium NCTC 135 + 0.15% 10 cps Methocel grew slowly and erratically over a long period of time, but these studies were made with a poorly controlled atmosphere which was later replaced by a superior continuously replaced atmosphere. The same cells adapted to growth on medium NCTC 135 + 10% horse serum grew moderately well and gave over 4-fold increases in population (up to 1.3 x 10^6 cells/ml and 1.5 x 10^9 cells in the culture). Successful subcultures were made and the subsequent growth curves were essentially identical.

A subsequent run using strain L cells growing in medium NCTC 135 + 10% horse serum with 100 mg percent glucose and later 300 mg percent glucose provided an increase of 0.26 x 10^6 cells/ml to 1.28 x 10^6 cells/ml. After 20 days of operation the culture was subcultured. Following the first subculture the population increased from 0.61 x 10^6 to 4.2 x 10^6 cells/ml in 13 days and the culture was split a second time. The culture, having a volume of 1460 ml at termination, contained 10 grams wet weight of cells. Thus in 40 days the original culture of 404 x 10^6 cells produced a total of 10.1 x 10^9 cells which were used in our nucleic acid research. The percentage fluid renewals per day ranged from 21% initially to 95% at the higher populations; the number of cells lost in the medium removal process was negligible but still subject to improvement in technology.

The growth curves have definitely shown the optimum initial inoculation to be approximately 0.56 x 10^6 cells/ml. This is very important information for all future studies. It is noted when medium NCTC 135 + 10% horse serum is used the rate of fluid renewal should be increased with increases in cell populations and the optimum percent of fluid renewal is that which maintains the concentration of glucose and lactic acid in the culture fluid between 50-60 mg percent and 30-40 mg percent respectively. It appears that good consistent logarithmic growth can be sustained indefinitely if the fluid renewal is sufficient to maintain these levels.

The results of several subsequent attempts to grow L-1210 ascites tumor cells in the identical culture system were not to our satisfaction for technical reasons. Growth of these cells under these culture conditions was excellent. The total loss of cells from carry-over through the withdrawal tube indicated that L-1210 cells must have a lower specific gravity than strain L cells. The new medium removal system, based on improving the centrifugal action has been developed to allow the culture of L-1210 cells as well as L cells with no loss of cells in the removal of exhausted media. A prototype has been fabricated from teflon and tested. Initial results were encouraging, but the teflon-to-teflon ball joint bearing galled at the high speeds found necessary for efficient cell removal. A high speed bearing has been designed and fabricated by an outside company. This bearing has been tested recently; it was found not to leak and has resulted in better separation of cells from the withdrawal fluid.

Significance: The development of these fluid renewal and withdrawal systems allows for the first time effective use of a continuously fed and continuously discharging, as opposed to an intermittent fed, mass suspension culture system. The resulting increase in amount of cells and better physiologic conditions, and the increased emulation of *in vivo* growth of tissue will allow superior experimental evaluation of cell metabolism before, during, and after the malignant transformation. Furthermore, it offers potential to explore the possibility of growing human diploid cells in fluid suspension--a much needed prototype for example in vaccine production. Conceivably, this culture system could be maintained indefinitely in any of the phases of growth and could allow valuable examination of the various aspects of basic growth metabolism that might be implicated in the transformation of normal cells to malignant cells. Further, it should be of paramount importance in the production of tissue to be used for virus production and sensitivity tests.

Proposed Course of Study: To test immediately improved rotor designs for separation of cells from fluid. This should, if successful, place us in a position to proceed with our objectives. Recently we have decided to try to incorporate in the suspension fluid a lightweight material on which cells can attach but still the combination can be kept in suspension. Certain very important cell types such as human diploids and non-neoplastic cells appear to be unable to grow in fluid suspension. By the means above we propose to try to propagate these for their obvious use in precise biochemical and biological study of conversion phenomena.

Serial No. NCI-3233 SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Cytogenetic studies of mouse embryo cells in vitro:

a. Comparative studies on the chromosomal characteristics in vitro of freshly explanted C3H mouse embryo cells in chemically defined medium NCTC 135 supplemented with either adult or fetal serum.

b. Chromosomal analysis of the transformability of a long-term, non-neoplastic C3H mouse embryo cell line in chemically defined medium NCTC 135 supplemented with 10% v/v fetal calf serum.

c. Computer chromosomal analysis of normal and neoplastic cells grown in vitro.

d. Comparative studies on the chromosomal characteristics and neoplastic transformations in vitro of freshly explanted C3H mouse embryo cells in chemically defined medium NCTC 135 supplemented with either horse or fetal calf serum: the effect of varying the periods of exposure to these two different serum supplements.

e. Chromosomal analysis of a non-neoplastic C3H mouse embryo cell line following exposure to the known carcinogen, 7,12-dimethylbenzanthracene (DMBA).

f. Chromosomal analysis of a non-neoplastic C3H mouse embryo cell line cultured in vitro in chemically defined medium NCTC 135 supplemented with 10% v/v fetal calf serum following adaptation to chemically defined medium NCTC 135 alone.

g. Chromosomal characteristics of C3Hf mouse embryo cells in vitro after short-term exposure to the carcinogens, 4-Nitroquinoline-N-oxide (4-NQO) and Methylnitrosourea (MNU).

Principal Investigator: J. T. Mitchell

Other Investigators: V. J. Evans, K. K. Sanford, F. M. Price, W. T. Andresen, J. L. Jackson, R. Cornell, and H. Kerr

Cooperating Units: Dr. W. T. Hall, NCI-3225; Dr. A. W. Pratt, H. Vreenegoor, M. Bruce, and L. Hodes, Data Systems Analysis Branch, Div. of Computer Research and Technology, NIH.

Man Years: See NCI-3211

Project Description (a): Studies on the chromosomal characteristics of freshly explanted C3H mouse embryo cells cultured in chemically defined medium NCTC 135 supplemented with either adult or fetal serum.

Objectives: 1) To compare the effect of various sera (horse, calf, fetal horse, fetal calf, and early fetal calf) on the chromosomal characteristics of freshly explanted C3H mouse embryo cells; 2) To correlate if possible any differences in chromosome morphology and mode with the onset of the neoplastic transformation phenomenon.

Methods Employed: Cell strains were established from 12-13 day C3H/HeN mouse embryos minced in chemically defined medium NCTC 135. The embryo mince was forced through a Swinney filter containing a 24 gauge monel screen. Each 2 ml of suspension contained 1 embryo. T-15 flask cultures were inoculated with 1-2 ml of this suspension and an equal amount of medium NCTC 135 supplemented with 10% v/v horse, fetal horse, calf, fetal calf, or early fetal calf serum (Flow Laboratories, Rockville, Md.) was added. Early fetal calf serum was obtained from a fetus at approximately 3 months gestation. The cultures were then gassed with 10% CO_2 in air and incubated at 37.5°C. Culture medium, 3 to 4 ml, was changed 3 times weekly and the cells subcultured at approximately 7 day intervals by gently mopping with perforated cellophane. The amount of nutrient medium, usually 3.0 ml, was determined by the growth and appearance of the cells and the pH shift of the culture medium. Two separate sets of 5 cell strains (or a total of 10 cell strains) were initiated from two different C3H/HeN mouse embryo cell pools. One set of 5 cell strains was carried by each of two investigators independently. Chromosome analyses were made on selected cultures by means of modifications of the procedure described by German et al. in J. Nat. Cancer Inst. 32: 681-707, 1964. One T-15 flask culture/cell strain was scraped and subcultured into one T-30 flask with 6 ml culture fluid. Two to three days later, following fluid renewal, 0.5 ml solution of 0.2 gamma/ml concentration of Colcemid (Ciba Pharmaceutical Co., Summit, New Jersey) was added directly to the culture flask for from 12-16 hours at 37.5°C to accumulate cells at the metaphase stage of mitosis. The cells were removed from the glass flask by gently mopping with a perforated cellophane strip. The supernatant medium was decanted into a centrifuge tube and the suspension centrifuged at 700 RPM for 5-7 minutes. Some clumping of cells resulted in the early generations. Hypotonic expansion of the cells was accomplished with distilled water, 0.1% sodium citrate, or 0.75 M potassium chloride. After the hypotonic exposure period of 20-30 minutes, one ml of freshly prepared fixative (absolute methanol + glacial acetic acid, 3:1) was slowly added and agitated for 5 minutes. The cell suspension was again centrifuged at 700 RPM for 8 minutes, the supernatant was withdrawn, and the packed cells resuspended with fresh fixative. This procedure was repeated two additional times to assure adequate fixation of the cell suspension. For air-drying, fresh fixative was added with agitation until the cell suspension appeared cloudy and any clumps were broken up. Two or three drops of cell suspension were placed on the microscope slide with a Pasteur pipette and allowed to flow down the length of the slide. The slide was shaken once or twice to remove excess fixative. The slide was allowed to dry completely and was later

stained with 2% aceto-orcein (Orcein, G. T. Gurr, London, England) for 2 hours. The slides were removed from the stain and passed rapidly through 3 changes of 95% ethanol, 2 changes of absolute ethanol, 1 change of absolute ethanol-xylene (1:1), 2 changes of xylene, and the coverslip mounted with Permount (Fisher Scientific Co.). In each preparation up to 50 well-spread metaphases were counted to determine the mode and range of chromosome numbers. These cells were analyzed for chromosomal abnormalities with particular emphasis on minutes, breaks, and biarmed chromosomes. In addition, other abnormalities, such as dicentrics, rings, long acrocentrics, chromatid interchanges, and endoreduplication were noted. Finally, estimates were made on 100 additional cells to confirm the randomness of the ploidy of the 50 cells selected for counting.

Major Findings: Chromosome preparations have been made on selected cultures from each cell line (Exps. 57-66) from 5 to 300 days in vitro. Complete chromosome analyses have been finished, and certain prevalent trends seem to be indicated in regard to ploidy distribution and the appearance of abnormal chromosomes. A rapid shift in the chromosome number away from actual diploidy (90% of the cells having a count of 40) was seen in cell strains supplemented with either horse or calf serum. The shift from diploidy was slower and more erratic in cell strains cultured in chemically defined medium NCTC 135 plus fetal sera. The chromosome modes of all cell strains did not become stabilized or well defined until almost 300 days in vitro. There seemed to be more abnormalities in cell strains in horse and calf serum than in these supplemented with fetal horse, fetal calf, and early fetal calf serum early in vitro. As time in culture progressed, this difference was diminished. Minute and biarmed chromosomes appeared slightly earlier and more frequently in cell lines supplemented with horse and calf serum, but eventually these abnormalities were observed in most cell lines irrespective of the serum supplement. Certain cell lines seemed to possess a specific chromosome marker, i.e., a minute, long acrocentric, or metacentric, but this phenomenon occurred at random irrespective of the medium. Those cell lines giving rise to tumors in isologous hosts did not present any clearer picture. Tumors were produced by cell lines supplemented with all five sera and time differentials were not particularly significant, although the fetal calf serum lines were the most delayed. Correlation of any differences in chromosome morphology and mode between any of these cell lines with the onset of neoplastic transformation is not possible. The differences observed between these lines (in fact, even between two cell lines supplemented with the same serum) seemed to reflect adaptation to an in vitro environment instead.

Significance: The significance of these findings does not lie in the possible differential effect between adult and fetal sera on the stability of chromosomes. It is clear that great variation of chromosome number and morphology can occur within one serum supplement even between two separate cell lines.

Chromosomes are generally considered the cellular site of genetic material and have been suggested as playing a role in the appearance of neoplastic properties of a cell. It has been shown previously in this laboratory that neoplastic conversion of mouse embryo cells cultured in vitro is delayed when fetal calf serum is used instead of horse serum to supplement chemically defined medium NCTC 135. Nevertheless, irregularities in chromosome number

and morphology have been seen in all cell strains observed in this investigation. Chromosomal abnormalities need not necessarily be a prerequisite for neoplastic growth, and cells becoming aneuploid or heteroploid and growing as continuous cultures may not be equivalent to transplantable neoplasias. One serum may select from the initial mixed population cells that are less vulnerable to structural alterations of chromosomal morphology, while another serum may promote more rapid propagation of potentially transformable cells. The occurrence of increased chromosomal anomalies may not be directly associated with neoplastic transformation, but may be influential in accelerating the genetic changes possibly required for the expression of neoplasia. Until extensive chromosome analyses have been completed, and more conclusive correlations between chromosomal changes (such as definite marker chromosomes - breaks, biarms) and the appearance of neoplastic tumors have been established, the specific role of the chromosome in neoplastic transformation in vitro cannot be determined.

Proposed Course of Study: A detailed evaluation of these experiments, differences, and/or similarities between each of the two cell lines on each of the five sera, and their effects on the chromosomal characteristics in relation to the neoplastic transformation process are being explored and prepared for publication.

Project Description (b): Chromosomal analysis of the transformability of a long-term, non-neoplastic C3H mouse embryo cell line in chemically defined medium NCTC 135 supplemented with 10% v/v fetal calf serum.

Objectives: 1) To determine changes in the chromosomal characteristics of cell line NCTC 4823 when switched from 10% fetal calf serum supplement to 10% horse serum, and to examine additional sublines of 4823 established in a variety of ways. 2) To correlate, if possible, any changes in chromosome morphology and mode with the onset of neoplastic transformation and the production of tumors.

Methods Employed: Cell strain NCTC 4823 was maintained under routine culture conditions in chemically defined medium NCTC 135 supplemented with 10% fetal calf serum for over 1000 days. One T-15 flask culture was then switched to chemically defined medium NCTC 135 supplemented with 10% horse serum, designated cell strain NCTC 5645, and maintained for several subculture generations. Since this subline has been established, several additional pairs of sublines have been set up in a variety of ways. The experimental procedures utilized (switching media, centrifugation of supernatent, virus infection, varied filtrations, etc.) have been described in NCI 3212, Project 1. Chromosome preparations were obtained from all cell lines at selected time intervals according to the procedure described earlier in this project (a). Chromosome analyses were carried out with particular emphasis on changes in morphology and mode. In addition, all cell lines were examined by Dr. W. T. Hall of the Laboratory of Biology, NCI, for the presence of detectable virus particles. All cell lines were inoculated into isologous hosts periodically to determine their tumor producing ability.

Major Findings: Cell line 4823 has been 100% heteroploid since 186 days in vitro, and possesses a nearly stabilized chromosome mode of 74-76; it contains abnormal chromosomes and has been characterized (Evans, et al., J Nat Cancer Inst 38: 761, 1967). NCTC 5645 after exposure to horse serum possessed 10% diploid cells. As time in horse serum increased to several months the diploid cell population rose to approximately 90%. A sharp modal value of 40 and a distinct minute marker chromosome are characteristic. Cells of 5645 implanted intramuscularly have consistently produced tumors histologically similar to lymphomas. NCTC 4823 has never been shown to possess morphologically identifiable virus particles, while 5645 contains mature and immature C-type particles both in inter- and intracellular spaces. The influence of these particles on the chromosomal characteristics and neoplastic properties of these cells is not clear.

Of the remaining sublines, all but one possess chromosome characteristics similar to 4823. The one exception, designated 5882, has a distinct chromosome rearrangement as seen by the presence of minutes, metacentrics, and a prominent decentric marker. All cell strains except two switches to 10% horse serum, 5716 and 5736, and 4823, have been shown to possess virus particles. Yet, none of these many cell lines is capable of producing tumors except 5645. Even though the presence of virus particles similar to those seen in 5645 has been demonstrated, these lines do not give rise to tumors.

Significance: It appears from these observations that the switch to horse serum has effected a classical cell selection from the NCTC 4823 cell population. This can only be inferred. Possibly horse serum has favored the multiplication of a cell type that was inhibited in NCTC 4823 in the presence of fetal calf serum. Horse serum could affect particular cells by altering surface membranes and, thus, give rise to a different population. Further confirmatory evidence for cell selection must wait for completion of several experimental approaches to this problem. From these results it is still difficult to evaluate the role of heteroploidy in the neoplastic conversion of cells in culture. There is no evidence at the present time that all heteroploid cell lines are tumorigenic or neoplastic. The heteroploid pattern appears to be associated more closely with the adaptation of cells to culture conditions than to their neoplastic or normal state. Proof of direct involvement of virus or virus-like particles in the initiation of "spontaneous" neoplastic transformation has not been demonstrated in our system. In fact, we have several neoplastic cell lines devoid of detectable virus, indicating that neoplastic transformation can occur independent of virus.

Proposed Course of Study: Completion and evaluation of existing experiments will result in the preparation of this study for publication.

Project Description (c): Computer chromosomal analysis of normal and neoplastic cells grown in vitro.

Objectives: This long-term projected study entails the following aspects: 1) To evaluate as the control the karyogram of the inbred mouse with a multiple component automated system including computer, scanner, and display units. This system will be developed by the Division of Computer Research and Technology, NIH. 2) To analyze if possible with this same system the chromosomes of

several strains of mouse embryo cells from the time of explantation through the neoplastic conversion. Emphasis will be placed on the following criteria in order to obtain the most helpful information:

a) Count of chromosomes
b) Measurement of perimeter
c) Measurement of overall length
d) Measurement of total area
e) Determination of centromere position
f) Measurement of length of short arms
g) Measurement of length of long arms
h) Ratio of arm lengths
i) Determination of number of breaks
j) Determination of position of breaks
k) Location of secondary constrictions and/or satellites

Methods Employed: Standard chromosome preparations of mouse embryo cells have been supplied from our studies to the Division of Computer Research and Technology. Both microscope slides and photomicrographs have been given to DCRT so that various scanning procedures and programs could be attempted.

Major Findings: To date, the problem of programming has been explored to a limited degree. A straight line logic program has been applied to mouse chromosome material and found to be inadequate as well as insufficient. In addition, direct scanning of photomicrographs has been attempted, utilizing equipment outside of the NIH, but results so far are too preliminary and fragmentary to warrant any sound conclusions. Major effort has been expended by DCRT in the design of a functional computer-display system that could adequately handle the above-mentioned criteria. This system has been submitted as a proposal, and final negotiations are underway for the awarding of a research contract to build the hardware required in this system. It is hoped that this system will be operational within a few months, and a computer-aided chromosome analysis program will be actively under investigation.

Significance: Minimal speculations can be made on the data obtained to date. The scope of this project is extensive and many more fundamental aspects must be worked out before meaningful information will be obtained. However, an automated computer system is certainly possible and desirable for these cytogenetic studies. The multitude of metaphase plates required for each critical analysis of an experimental variable will demand the refinement of this automated system. Eventually, thousands of cells will be automatically scanned and accurately analyzed in a few minutes, replacing the present time-consuming and tedious manual procedures. Hopefully, any of the knowledge gained through these computer techniques could be applied to abnormal human chromosome situations associated with specific disorders.

Proposed Course of Study: Additional slides and photomicrographs of mouse chromosome spreads will be made available to the Division of Computer Research and Technology. In particular, chromosomes showing breaks and secondary constrictions, as well as chromosome overlap situations, will be investigated for the possible application of various modified programs and scanning procedures

with the new system. A continuation of pertinent discussions with DCRT on this project will be carried out.

Project Description (d): Studies on the chromosomal characteristics and neoplastic transformations in vitro of freshly explanted C3H mouse embryo cells in chemically defined medium NCTC 135 supplemented with either horse or fetal calf serum: the effect of varying the periods of exposure to these two different serum supplements.

Objectives: 1) To compare the effect of varying the periods of exposure to either horse serum or fetal calf serum on the chromosomal characteristics of freshly explanted C3H mouse embryo cells. 2) To correlate, if possible, any changes in chromosome morphology and mode with the onset of neoplastic conversion, and therefore possibly reveal information concerning the mechanism(s) responsible for this phenomenon.

Methods Employed: Four separate sets of eleven cell lines were recently established from twelve to thirteen day old C3H/HeN mouse embryos using routine laboratory culture techniques (see a). Several T-flasks were set up in medium NCTC 135 with either 10% horse serum or 10% fetal calf serum as the serum supplement. After 1, 2, 4, 6, 8, 11, 13, 15, or 30 days of cultivation in 10% horse serum, sublines were established by switching to 10% fetal calf serum. The eleven cell lines/set (2 in control media, 9 switched sublines) have been maintained by the usual fluid renewal and subculture laboratory procedures. Some chromosome preparations were made on the majority of these cell lines during the first few days of cultivation, but to date no animal injections have been done.

Major Findings: No chromosome analyses have been completed on these cell lines, and it is too early to draw any conclusions at this time. All cell lines will be inoculated into isologous hosts in the near future, and hopefully, will reveal pertinent information regarding their neoplastic potential.

Significance: Prior switch studies in this laboratory have shown that horse serum and fetal calf serum have differential effects on neoplastic conversion of cells in vitro. In general, horse serum promotes while fetal calf serum delays or prevents this phenomenon. It has been demonstrated that as little as fifteen days' exposure to horse serum prior to subsequent cultivation in fetal calf serum will result in the neoplastic conversion of cells in vitro. The purpose of this experiment is to determine the minimal exposure to horse serum required, and to attempt to define within a day or few days when this transformation phenomenon might be initiated within this in vitro system. It is anticipated that cytogenetic analyses might reveal differences between some of these lines at the chromosomal level, and therefore, possibly mark a cell type(s) involved in neoplastic transformation. Correlation of changes in chromosome morphology and mode with the onset of neoplastic conversion will be attempted.

Proposed Course of Study: Comparative chromosome analyses will be completed on all four separate sets of eleven cell lines at various periods of cultivation. Particular emphasis will be placed at those times when the

animal injections are carried out. Analyses will also be done on any tumors produced.

Project Description (e): Chromosomal analysis of a non-neoplastic C3H mouse embryo cell line following exposure to the known carcinogen, 7,12-dimethylbenzanthracene (DMBA).

Objectives: 1) To determine whether exposure of a long-term, non-neoplastic C3H mouse embryo cell line to the known carcinogen, 7,12-dimethylbenzanthracene (DMBA), has altered the chromosomal characteristics of this line. 2) To ascertain, if possible, whether any newly altered chromosomal component might be involved with any resulting neoplastic transformation.

Methods Employed: Cell line NCTC 4823 had been maintained under routine culture conditions in chemically defined medium NCTC 135 supplemented with 10% fetal calf serum for over 1000 days when one flask culture was exposed to DMBA. The concentration of DMBA used, length of exposure, subsequent changes in the culture, and required maintenance conditions are described in NCI 3212, Project 2. The cell line exposed to DMBA, designated NCTC 6049 and 6182, has been maintained for several subculture generations. Chromosome preparations were obtained from both 6049 and 6182 in the second subculture generation according to the procedure described earlier in this project (a). Chromosome analyses were carried out with particular emphasis on changes in morphology and mode and compared with 4823.

Major Findings: Cell line NCTC 4823 has been 100% heteroploid since 186 days in vitro, and presently possesses a rather sharp hypotetraploid mode of 74-76 chromosomes with 1-2 minutes/cell. Cell lines 6049 and 6182 are quite identical to each other, but very different from 4823. They are mixoploid cell lines, possessing about 75% hyperdiploid and 25% hypertetraploid cells. In addition, these cells have 1-3 minutes and several biarmed chromosomes/cell. These lines are also bimodal, having one chromosome mode at 54-57 and the other at 103-108. No chromosomal characteristics similar to 4823 have been observed in either 6049 or 6182, and it seems as if exposure to DMBA has altered the chromosomes of cell line 4823.

Significance: After only one set of observations, it is rather difficult to evaluate the significance of these results. Yet, it does appear that 4823 exposed to DMBA has in some way been drastically altered, at least in its chromosome components. Since no cell progenitors from 4823 have been observed in either 6049 or 6182, one cannot say that cell selection has occurred in this case. It seems more reasonable to speculate that DMBA exerted a pharmacological effect on 4823 cells. This could have resulted in mutagenesis, with different cell types eventually surviving. As these new altered cells replicated, cell selection could have occurred with the eventual domination of one or two cell types. It remains to be seen if these new cell types, present in 6049 and 6182 and absent in 4823, have been transformed and can produce tumors. If they have been, one might conclude that 4823 possesses a cell type(s) capable of being transformed and producing tumors.

Proposed Course of Study: Additional chromosomal analyses will be performed on these altered cell lines as time in vitro proceeds. More animal inoculations will be done, and any resulting tumors will be analyzed both in vivo and in vitro in an attempt to correlate specific chromosomal changes with tumor production.

Project Description (f): Chromosomal analysis of a non-neoplastic C3H mouse embryo cell line cultured in vitro in chemically defined medium NCTC 135 supplemented with 10% v/v fetal calf serum and subsequently adapted to chemically defined medium NCTC 135 alone.

Objectives: 1) To ascertain whether adaptation of a long-term, non-neoplastic C3H mouse embryo cell line, cultured in vitro in chemically defined medium NCTC 135 + 10% fetal calf serum for about three years, to chemically defined medium NCTC 135 alone will affect or alter the chromosomal characteristics of this line. 2) To determine if a transformable cell type(s) can be selected out from the mixed cell population of NCTC 4823 by this adaptation procedure.

Methods Employed: The in vitro adaptation of cell line NCTC 4823 to chemically defined medium NCTC 135 alone is described in NCI 3212, Project 1. This procedure has resulted in the establishment of at least three separate and different sublines, designated 6000, 6071, and 6196, which have been maintained for several subculture generations. Chromosome preparations were obtained from each of these sublines at selected periods of in vitro cultivation according to the procedure described earlier in this project (a). Analyses were carried out with emphasis on chromosome alterations in morphology and mode and compared with 4823.

Major Findings: Preliminary findings have indicated that cell lines 6000, 6071, and 6196 are different from one another, and quite different from cell line 4823 with respect to chromosome mode and morphology. These results can best be demonstrated by the following table:

CELL LINE	MODE	ABNORMAL CHROMOSOMES/CELL			
		Minutes	Biarms	Breaks	Other
4823	74-76 (100%)	1-2 (90%)	-- (0%)	-- (0%)	-- (0%)
6000	46-47 (12%)	.7	1.2	.1	--
	74-76 (88%)	(68%)	(38%)	(8%)	(0%)
6071	47-55 (77%)	.5	2.5	.1	--
	74-76 (23%)	(50%)	(80%)	(8%)	(0%)
6196	52-57 (65%)	.5	2.0	.4	Long Acrocentric 1.0
	74-76 (35%)	(60%)	(90%)	(35%)	(60%)

It can be seen that the sublines 6000, 6071, and 6196 are bimodal and possess different percentages of the 4823 cell types. In addition, they are further different from one another in that they have varied abnormal chromosomes. None of these sublines possess the same chromosome characteristics of 4823, and yet, each has a certain percentage of its population similar to 4823-type cells. It appears as if the adaptation of 4823 to chemically defined medium has resulted in the selection and alteration of particular cell types. It is still too early for any of these lines to have produced tumors, so the tumor potential of these new cell types is still unknown.

Significance: It is difficult to discuss the significance of these results until many more observations have been analyzed, and the tumor-producing potential of these adapted sublines has been determined. As NCTC 4823 was gradually adapted to chemically defined media, it is conceivable that less and less serum concentrations may have released a repressor-like substance and thus allowed the replication and expression of a previously undetected cell type. Since different adaptative procedures were utilized, different "signals" could have resulted, thus accounting for variations seen in the different chromosome characteristics. The identification and isolation of such a substance or substances would be difficult, but could contribute much information as a possible factor in the neoplastic transformation phenomenon.

Proposed Course of Study: As these chemically adapted cell lines are carried in vitro longer, additional chromosome analyses will be done periodically to characterize their stability and particular features. Any resulting tumors will be analyzed both in vivo and in vitro in an attempt to correlate specific chromosome aberrations with tumor production.

Project Description (g): Chromosomal characteristics of C3Hf mouse embryo cells in vitro after short-term exposure to the carcinogens, 4-Nitroquinoline N-oxide (4-NQO), and Methylnitrosourea (MNU).

Objective: To determine the effect, if any, on the chromosomes of C3Hf mouse embryo cells cultured in vitro after short-term exposure to the carcinogens, 4-Nitroquinoline N-oxide (4-NQO), and Methylnitrosourea (MNU).

Methods Employed: Routine procedures for establishing the C3Hf mouse embryo cells in culture were utilized [see this project (a)]/ After about four-six weeks in vitro, cultures were exposed for one hour to several concentrations of each of the two carcinogens, 4-NQO and MNU, and maintained under standard conditions. The details of this portion of this experiment can be obtained from NCI 3213, Project 1. At selected time periods after exposure to the carcinogens, control and experimental cultures were prepared for both chromosomal analysis and animal inoculation for tumor production. The chromosome preparations were obtained according to the procedure described earlier in this project (a).

Major Findings: To date, no complete chromosome analyses have been performed on these cell lines, but random scanning of the chromosome preparations have revealed little or no differences when the experimental cell lines are compared with control cell lines. This may be due to inadequate exposure

of the cells to the carcinogens, but it is too early to make any definite conclusions. Detailed and precise chromosome analysis will have to be carried out before any significance can be attributed to these preliminary observations. It is still too early for any of the injected cell lines to have given rise to tumors.

Significance: It had been anticipated that a specific chromosomal aberration or aberrations might result from exposure of C3Hf mouse embryo cells to these two carcinogens, 4-NQO and MNU. If tumors were then obtained from some of these lines, a possible link between a known carcinogen, a specific cytogenetic marker, and tumor production might be available. However, much work remains to be done before this can be realized and we can utilize this system in this way as a model system for the study of neoplasia.

Proposed Course of Study: Additional chromosome analyses and animal injection studies are being carried out. After completion and evaluation of these existing experiments, any effect on the chromosomes of these cells will be determined. At that point future studies involving these carcinogens will be outlined and pursued.

Publications:

1) Evans, V.J., Jackson, J.L., Andresen, W.F., and Mitchell, J.T.: Chromosomal characteristics and neoplastic transformation of mouse embryo cells in vitro in horse and fetal calf serum. J Nat Cancer Inst 38: 761-769, 1967.
2) Mitchell, J.T.: Studies with thalidomide and related compounds on early developing mouse embryos. Dissert.Abs. 27: 4177-4178, 1967.
3) Mitchell, J.T., Price, F.M., and Evans, V.J.: Chromosome studies of mouse embryo cell lines. I. Analysis of a tumor producing cell line derived from long-term, non-neoplastic cells cultured in vitro. J Cell Biol 35: 95A, 1967.

Honors, Awards, and Other Activities: J. T. Mitchell

1) Received Founder's Day Award, New York University, 1967, for high scholastic achievement during graduate studies.
2) Received Graduate School of Arts and Science Alumni Association Jay F. Krakauer Award, New York University, 1967, awarded annually to the graduate student submitting the outstanding thesis in the field of science.
3) Presented paper at the 7th Annual Meeting of the American Society for Cell Biology held in Denver, Colorado, November 13-15, 1967.
4) Presented seminar at Department of Biology, Graduate School of Arts and Science, New York University in December, 1967.

NCI Serial No. 3234-A SAME
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Primary biochemical alterations leading to neoplastic conversion.

Principal Investigator: R. R. Gantt

Other Investigators: V. J. Evans, K. K. Sanford, and J. T. Mitchell

Project Description: A program designed to elucidate the primary chemical alterations which are necessary biochemical changes leading to neoplastic transformation.

Objectives: To apply the various biochemical techniques to a system developed in the Tissue Culture Section (see NCI Serial No. 3211) in order to identify and understand the chemical events leading to neoplastic transformation. There are three immediate objectives: (1) To identify the causal agent (or agents) responsible for the dramatic difference between tissue culture media containing fetal calf serum and medium containing horse serum (see NCI Serial No. 3212); (2) To identify primary changes in the nucleic acids and proteins during neoplastic transformation; and (3) To compare in a general way the physical and chemical changes in the membrane components during transformation.

Methods Employed:

(1) In order to identify the agent (or agents) responsible for the difference in fetal calf serum and horse serum a series of parallel fractionations have been carried out. These fractions were prepared by ultrafiltration through a colloidon membrane, Sephadex gel filtration, and ammonium sulfate precipitation, and they are being tested for their ability to transform cells or to protect the cells from transformation.

(2) The identification of the primary changes in the nucleic acids is carried out by first establishing differences before and after neoplastic conversion and then to correlate these differences with the conversion time and with the means of conversion (i.e., viruses, chemical carcinogens, or the horse serum—fetal calf serum system). These differences are monitored by double isotope-co-elution chromatography and enzyme analysis.

(3) The methods employed for detecting changes in the physical and chemical properties of the membranes of paired neoplastic and non-neoplastic cell lines is limited in scope at this time. A specific glycoprotein agglutinin was examined to determine if its activity demonstrated the same correlationof activity versus malignancy and an *in vitro* system as it showed in *in vivo* systems.

Major Findings:

(1) Fetuin, a glycoprotein isolated from fetal calf serum and not present in horse serum, is not responsible for inhibiting the neoplastic transformation of normal cells (see NCI Serial No. 3212).

(2) A qualitative difference as well as a quantitative difference in the RNA methylase complements of paired neoplastic and non-neoplastic cells cultured in vitro has been established.

(3) A glycoprotein present in wheat germ has been characterized (Burger and Goldberg, PNAS 57: 359, 1967) and reported to be a specific agglutinin for malignant cells. We established that with our cultures grown in vitro there is no correlation between malignancy and agglutination activity, and further, there is no other obvious correlation such as the media used, age of the culture, or whether it was grown in suspension or as a monolayer.

Significance and proposed course of investigation:

(1) The fact that fetuin, isolated from ammonium sulfate precipitation, failed to inhibit neoplastic conversion rules out the major difference between fetal calf serum and horse serum as the responsible agent. A series of experiments have been set up to localize the agent in the large molecule fractions or the small molecule fractions of the serum; the fractionation is complete and the cultures are set up for assay. In addition to fractionation studies, specific protein utilization will be assayed in order to determine which specific protein fraction should be examined to localize the difference in the serums. This will be done by comparing depleted medium with unused medium on analytical polyacylamide electrophoretic gels. The specific proteins which are depleted will be isolated from fresh serum and their efficacy for both promoting growth and inhibiting transformation will be tested.

(2) The finding of a qualitative difference in the RNA methylases in our in vitro system of paired neoplastic and non-neoplastic lines is important because this provides an approach for the study of the relationship of nucleic acid methylation to oncogenesis. We are now in a position to relate the changes in methylases to their specific substrates, the transfer RNA's, and ascribe biological functions to these changes and determine whether or not these changes correlate with neoplastic conversion in the horse serum--fetal calf serum system and in addition by viral and chemical carcinogens. Further, insight into the problem of whether or not neoplastic cell lines are a mixture of malignant cells and non-malignant cells can be studied by hypermethylation. Since one can add methyl groups to normal nucleic acids by using methylases from neoplastic cells, in principle we should be able to grow a neoplastic cell line on cover slips, extract the methylases from the same culture grown in T-60 flasks, and hypermethylate the cover slip cells. Any normal cells present with the neoplastic cells should have nucleic acids which accept tritiated methyl groups from s-adenosylmethionine which could then be detected by radioautography.

(3) The lack of a correlation between malignancy and agglutination clearly shows that the presence of this specific site is not related to neoplasia even though the in vivo systems correlate with a couple of exceptions. It

was hoped that this reported tumor-specific site would permit a rapid assay for neoplastic conversion to supplement the injection assay system. Membrane studies will be continued, comparing chemical and physical properties to attempt to find a correlation between neoplastic transformation and membrane properties. Such a correlation would be most desirable in order to assay for transformation quickly, but in addition, it is important to the general problem of membrane structure and malignancy.

NCI Serial No. 3237, Project 1 NONE
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project 1 Title: Studies of ultrastructure, cytochemistry, neoplastic transformation and viral content of mammalian cells in vitro.

a. Neoplastic transformation in vitro: effects of varying the periods of exposure to two different serum supplements.

b. Ultrastructural and cytochemical studies of:
1. neoplastic and non-neoplastic cells in vitro derived from identical minced embryo pools
2. tumors derived from the neoplastic lines
3. explants of the tumors

c. Ultrastructural and cytochemical studies of long-term, non-neoplastic cell line 4823 and lines derived from it which have been:
1. transformed with exposure to 10% horse serum
2. treated with the carcinogen DMBA
3. adapted to chemically defined medium

d. Ultrastructural study of transmission of murine tumor viruses from mother to offspring by placental and intestinal routes.

Principal Investigator: R. Cornell

Other Investigators: V. J. Evans, and J. T. Mitchell

Cooperating Units: V. T. Marchesi, P. T. Mora, Macromolecular Biology Section, NCI

Project Description (a): Studies on neoplastic activity of C3H mouse embryo cells cultured either: continuously in 10% horse serum or 10% fetal calf serum for 180 days before injection; or 1, 2, 4, 6, 8, 11, 13, 15, or 30 days of cultivation in 10% horse serum and then transfer to 10% fetal calf serum for the remainder of 180 days.

Objectives: To define the minimal amount of horse serum exposure adequate for the onset of neoplastic transformation to occur.

Methods Employed: On four separate occasions cell strains have been established from 13 and 14 day C3H/HeN mouse embryos in the manner which has become standard for this laboratory. Each of these four times, a portion of the minced embryo

pool was placed in either 10% horse serum or 10% fetal calf serum, and then, where appropriate, switched from horse serum to fetal calf serum according to the above schedule. The cultures have subsequently been maintained by our usual procedures of gassing with 10% carbon dioxide in air, incubation at 37.5°C, changing of media three times a week, and subculturing at approximately seven day intervals by mopping with perforated cellophane.

Major Findings: At this time it is too early in the course of this experiment to draw any conclusions. In several weeks' time, the initial animal injections will begin, and it is only after that is done that significant information will accrue.

Significance: From prior studies in this laboratory, it has been shown that horse serum and fetal calf serum have divergent effects on spontaneous neoplastic transformation in vitro, the horse serum permitting early transformation and the fetal calf serum retarding the transformation. It has also been shown that embryonic cells need not be cultured continuously in horse serum for the promoting effect to be effective. Cells with as little as 15 days' exposure to horse serum have become capable of producing neoplasia even after being subsequently cultivated in fetal calf serum. It is the purpose of this experiment to determine the minimal exposure to horse serum required for the promoting effect. Such information will greatly enhance the utility of our system as a model system for the study of neoplasia. More precise information about the time of transformation should enable us to study that phenomenon in much greater detail.

Proposed Course of Study: We are now in the process of building up these 44 cell lines for animal injection studies. Animals so injected will be observed for one year's time to see if tumors are produced. Any tumors so produced will then be explanted and cultivated in vitro and subsequently re-injected to check their neoplasticity.

Project Description (b): Electron microscopic and cytochemical study of the cell lines described in (a), with particular emphasis on the "control" lines, i.e., those lines cultivated continuously in 10% horse serum or 10% fetal calf serum.

Objectives: To identify any ultrastructural changes associated with neoplastic transformation within this model system, including the presence or absence of viral particles. To correlate if possible any differences in the distribution of cytochemically demonstrable enzymes and other cell constituents with the presence of neoplastic transformation.

Methods Employed: For electron microscopy, standardized techniques have been followed of glutaraldehyde fixation followed by buffered osmium tetroxide fixation and Epon embedding. Cytochemistry is performed on coverslip preparations fixed in buffered glutaraldehyde and stored in cold cacodylate buffer, pH 7.4.

Major Findings: To date no distinctive ultrastructural or cytochemical difference has been detected between cells cultured continuously in horse serum

and fetal calf serum. Viral particles have not been observed. A highly distinctive feature of both groups of cells is the presence of enormously dilated rough endoplasmic reticulum.

Proposed Course of Study: At the time of animal injection each cell line will be prepared for detailed examination, and any tumors derived from these lines will also be studied.

Project Description (c): Ultrastructural and cytochemical study of 4823, a line cultivated 4 years on fetal calf serum, which fails to yield tumors. This line is to be compared with lines derived from it which have been grown on horse serum, exposed to DMBA and cultured on fetal calf serum, adapted to medium NCTC 135 without serum supplement.

Objectives: To compare the effect of these various treatments with the status of the control line. A companion study includes testing these lines for neoplasticity and any tumors derived from that study will also be examined.

Methods Employed: As above.

Major Findings: It has so far been determined that there are two distinctive types of cells in 4823, using morphological criteria. One is a large fibroblastic cell, whose most characteristic feature besides its overall shape is the presence of enormously dilated rough endoplasmic reticulum. The second cell type is a smaller, rounder cell, whose most characteristic feature is the presence of a dense coat of microvilli. These projections from the cell surfaces have an abundance of fibers at their core, and these fibers are linked to a similar cytoplasmic network of fibers. Treatment with DMBA and adaptation to chemically defined media have favored abundant growth of a cell type which may be similar to this second cell type, and although 4823 contains mostly cells of the fibroblastic type, the derivative lines consist almost entirely of rounder cells with a microvillous coat. An incidental benefit of the derivative lines is the opportunity to study microvilli and the substructure in greater detail than is usually afforded in tissue culture cells. Viral particles have not been observed.

Significance: Without knowledge of the presence or absence of neoplastic potential in these lines, it is difficult to determine the precise significance of these preliminary findings.

Proposed Course of Study: Any tumors derived from these lines will also be studied, as well as any lines derived from tumor explants. Moreover, in order to define whether or not the changes noted in chemically defined media are stable, specimens from these lines will be switched to 10% fetal calf serum and then re-examined. A more detailed study of the microvilli will also be made, with special emphasis of the substructure and organization of the fibrous network of which they are composed.

Project Description (d): A morphological and cytochemical study of yolk sac and nursling intestine of C3H/HeN mice.

Objectives: To attempt to identify possible modes of transmission of murine

tumor viruses from mother to offspring. Our mouse embryo lines frequently have shown the presence of viral particles in the past, and to date the mode by which these particles appear in embryonic tissue has not been identified. By the same token, it is not precisely known to what extent, if any, viruses in milk can be transmitted by intestinal tissue in the neonatal period.

Methods Employed: Electron microscopy.

Significance: Results from this planned study may reveal important information about mechanisms by which tumor viruses are disseminated in the perinatal period.

Proposed Course of Study: After initial screening of the tissues under study, known oncogenic viruses will be administered to determine their areas of transmission. It is anticipated from previous work of the principal investigator and recent work of Silverstein and Dales, that phago-lysosomes have a role in the phenomenon under investigation. Therefore, the study will also include performance of suitable cytochemical techniques at the ultrastructural level.

NCI Serial No. 3237, Project 2 NONE
1. Laboratory of Biology
2. Tissue Culture Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project 2 Title: Studies of surface membranes of mammalian cells.

a. Modification of the Wachstein and Meisel lead salt method for the localization of Mg-Na-K-activated adenosine triphosphatase of the cell surface.

b. Isolation and characterization of the cell surface reversible stabilization with fluorescein mercuric acetate.

Principal Investigators: R. Cornell, V. T. Marchesi

Other Investigators: V. J. Evans, P. T. Mora

Cooperating Units: Macromolecular Biology Section, NCI

Project Description (a): Quantitative measurement of the inhibitory effect of aldehyde fixation and varying concentrations of the capture reagent lead nitrate. Lowering the concentration of lead and fixative sufficiently to permit performance of cytochemical tests under conditions of minimal inhibition of enzymatic activity.

Objectives: To determine site of inorganic phosphate release which is due to Na-K-Mg activated ademosine triphosphatase activity of cell surfaces.

Methods Employed: ATPase assay and cytochemical demonstration of ATPase activity according to the methods of V. T. Marchesi and G. E. Palade (The Journal of Cell Biology 35: 385, 1967).

Major Findings: Thus far, quantitative assay on highly purified brush border of small intestine has demonstrated the presence of Mg-Na-K activated ATPase.

Significance: This ATPase is either part of or functionally associated with the mechanism of active transport of Na and K. Although evidence has accumulated associating this enzymatic function with cell membranes, cytochemical methods other than the Marchesi-Palade approach are unsatisfactory because of either destruction by aldehyde fixation or inhibition by the heavy metal used as capture reagent.

Proposed Course of Study: Because of the relative ease of isolation this study has begun with use of intestinal brush border. Dependent on evaluation of current experiments with brush border, these methods will be applied to

tissue culture cells using methods for isolation of surface membranes as described below.

Project Description (b): Studies on isolation and characterization of cell surfaces isolated after stabilization with fluorescein mercuric acetate (FMA) as introduced by L. Warren and associates.

Objectives: 1) To determine if FMA did in fact stabilize cell surfaces and thereby protect them from mechanical trauma during isolation procedures; 2) To determine if the effect of FMA is reversible; and 3) To determine if a previously characterized protein constituent of cell surfaces could be isolated in antigenically intact form after treatment of cell membranes with FMA.

Methods Employed: To test the effects of FMA on cell surfaces, red blood cells were selected because of the ease of obtaining them in relatively pure, large amounts. The FMA was used as a saturated solution in 0.02M Tris buffer pH 7.5.

Major Findings: It has been determined that red cells previously treated with FMA have markedly decreased mechanical fragility. However, they have a marked increased osmotic fragility and in fact lyse rapidly even in hypertonic solution. It has been found in addition that treatment with FMA exerts an inhibitory effect upon membrane adenosine triphosphatase. Addition of mercaptoethanol in very dilute solution can rapidly reverse the stabilizing effect of FMA. Moreover, it was also determined that under low ionic strength conditions in the presence of mercaptoethanol, a specific membrane protein, spectrin, could be isolated in antigenically intact form.

Significance: Though introduced earlier for purposes of isolating surface membranes, the effect on membrane stabilization had never been documented in the past. Moreover, in the course of our studies, it has been determined that the decrease in mechanical fragility is reversible with mercaptoethanol, and these findings should make FMA stabilization an all the more attractive agent for studies on nucleated cells. In addition, the fact that FMA treated cells, even in hypertonic solution, undergo rapid lysis should facilitate separation of surface from underlying cytoplasm.

Proposed Course of Study: The detailed study of the membranes of the cell is still a relatively underdeveloped field compared with other areas of biochemical investigation. A fundamental reason for this has been the lack of good techniques for isolating and purifying cell surfaces for biochemical analysis. Tissue culture cells represent a particularly good source of mammalian cells for such studies, and the mouse embryo lines in our laboratory represent a particularly good source for investigations aimed at characterizing fundamental differences in the surfaces of neoplastic as opposed to non-neoplastic cells. In our future investigations, we shall utilize the FMA technique for isolating and purifying cell surfaces and shall attempt to correlate electron-microscopic examination with biochemical and immunological analysis of the isolated material.

SUMMARY STATEMENT
LABORATORY OF PATHOLOGY
PATHOLOGIC ANATOMY BRANCH
CL&C, NCI
July 1, 1967 - June 30, 1968

INTRODUCTION

The work in the Laboratory of Pathology is separated into two general areas, which are the Department of Pathologic Anatomy and the Laboratory of Pathology. The staff of Pathologic Anatomy performs the autopsies, examines the biopsies and surgical pathology specimens and does exfoliative cytology for patients in the Clinical Center. The annual report on the number of autopsies, biopsies, and cytodiagnostic examinations is attached.

Another important operation maintained in the Laboratory of Pathology is the Pathological Technology Section. A report prepared by the head of this section, Mr. Joseph Albrecht, describes the work performed in this section.

The primary aim of the staff of the Laboratory of Pathology is to carry out experimental cancer research. For this a variety of techniques and experimental animals are used. Each senior staff member carries out his own experiments, but two or more members within the Laboratory may collaborate and individual members collaborate with many others in the National Cancer Institute, and the National Institutes of Health.

There is free exchange of information and assistance between and among all the pathologists in the Laboratory. While the Annual Report reflects in general the work of the two groups, it also shows that there is considerable interchange. Most of the members of the Department of Pathologic Anatomy have under study one or more problems involving cancer in man, or some other disease in man, that they carry out either independently or in collaboration with clinicians. The autopsies and the surgical pathology specimens and biopsies are examined with the greatest of care. They are initially studied by residents and their diagnoses are all reviewed by senior staff members.

Most of the members of the Department of Pathologic Anatomy also carry out experimental research using animals, tissue culture and other special techniques. The residents in Pathologic Anatomy are particularly ambitious in carrying out research programs.

EXPERIMENTAL RESEARCH

The experimental work in the Laboratory of Pathology is not restricted to a single project, or to a group of closely related projects, but each pathologist follows his particular line of interest and training. The projects range from I. Studies on cancer in man such as a review of bladder cancer in New Orleans, lung cancer in World War I veterans and gastric cancer in Japanese migrants, and related studies of cancer in animals where possible carcinogenic hazards to man are tested, through II. Studies in which the pathogenesis of animal tumors is traced, and the biologic behavior of transplantable tumors is recorded, to III. Studies at a cellular or molecular level using a variety of techniques in immunology, tissue culture, electron microscopy and virology to explore fundamental problems of the cancer cell and its interaction with normal tissue. In addition, there are IV. Collaborative projects where the training of a pathologist is indispensable, and V. Special contributions and recognition to members of the staff.

Many of the projects are continuations of work described in last year's report, but progress has been made in many areas. Some projects have been completed and the results published, and many new experiments have been started. For convenience in preparing this year's summary report, the outline given below will be followed. Details of the projects may be found in the individual reports, and in the published papers from the Laboratory of Pathology. Although much of the work has been done in collaboration, only the name of the principal investigator is mentioned.

I. Cancer in Man, and Related Animal Studies

A. Geographic Pathology, Distribution of Cancer in Geographic Areas and Special Population Groups

Maps (Dunham)
Bladder Cancer in New Orleans (Dunham)
Uterine Cancer in the Negro in New York City and the Washington Metropolitan Area (Dunham)
Lung Cancer in World War I Veterans (Herrold)
Lung Cancer in LaPlata, S.A. and Bristol, England (Herrold)
Mesothelioma (Herrold)
Gastric Cancer in Japanese Migrants (Herrold)

B. Related Animal Studies

1. Tests of Possible Carcinogenic Substances in the Human Environment

Components of Betel Quid in the Hamster Cheek Pouch (Dunham)
Water Pollutants (Dunham)
Heated Fat (O'Gara)
Plant Substances (O'Gara)
Aflatoxin (Snell)
Enovid (Dunn)
Myleran (Chu)
Asbestos (Stanton)

2. Modifications of Carcinogenic Activity

Chemical Carcinogens and Vitamin A (Chu)

II. Cancer in Animals

A. Induction and Pathogenesis by Known or Suspected Carcinogens

Nitrosamines in Hamsters (Herrold)
Lung Tumors in Hamsters (Herrold)
Methylnitrosourea in Rats (Snell)
Bone Tumors in Rats by Cupric-Chelated Compound (Stanton)
Metal Carcinogenesis (O'Gara)
Testing of Dietary Factors and Combinations of Carcinogens (Mulay)
Lung Tumors in Rats (Mulay)

B. Data on Various Animal Species and Spontaneous and Induced Tumors in These Species

Mouse (Dunn)
Rat (Stewart-Snell)
Mastomys (Stewart-Snell)
Monkeys (Stewart-Snell)
Monkeys (O'Gara)
Invertebrate and Lower Vertebrates (Dawe)

C. Transplanted Tumors

Mouse (Dunn)
Rat (Stewart-Snell)
Contagious Tumor of Hamster (Banfield)
Hepatoma in a Primate (O'Gara)

III. Studies at Cellular or Molecular Level

A. Cytogenetics (Chu)

B. Tissue Culture of Hepatoma (Dawe)

C. Immunocytology

Application of Techniques (Malmgren)
Immunologic Factors in Carcinogenesis (Malmgren)
Mechanisms of Action (Berard)

D. Electron Microscopy

Technical Advances with Electron Probe and Scanning Electron Microscope (Banfield)
Correlated Study with EM on an Hormonally Responsive Epithelium (Grimley)
Collagenous Connective Tissue (Banfield)
Neuroendocrine Tumors (Grimley)
Cells from Human Tumors (Grimley)
Characteristics of Bone Tumors (Stanton)

E. Ultrastructural Research on Viruses

Infant Diarrhea of Mice (Banfield)
Elementary Body in Shope Fibroma (Banfield)
Arbovirus Studies (Grimley)
Herpes Simplex Cycle (Grimley)
Murine Sarcoma Virus (Stanton)

F. Polyoma Virus Tumors in Mice - Interrelation of Epithelium and Mesenchyme (Dawe)

G. Viral Carcinogenesis

SV40 and Oncogenic Human Adenovirus (Rabson)
Search for a Virus in Human Lymphomas (Rabson)
Cervical Lesions in Hamsters and Herpes Virus (Chu)
Murine Sarcoma Virus (Stanton)

H. Basic Virus Research and Interferon

Replicative RNA Forms of an Arbovirus (Friedman)
Production of Interferon by Non-Viral Substances (Friedman)
Possible Role of Interferon in Viral Oncogenesis (Friedman)

IV. Collaborative Research

Anterior Chamber Transplants (Dunn)
Induced Ovulation by Hormones (Powell)
Experimental Emphysema (Powell)

V. Special Contributions, and Recognition to Members of Staff

A. Editorial

B. Officials in Organizations

C. Participation in Meetings and Symposia

D. Services on Committees, Contracts, Etc.

I. Cancer in Man, and Related Animal Studies.

Since all but one of the members of the professional staff of the Laboratory of Pathology and Department of Pathologic Anatomy are medically trained, many research activities are correlated with the problem of human cancer. Several projects are directly utilizing human material, and others are using animals to test substances in the human environment that may be carcinogenic.

A. Geographic Pathology: Distribution of Cancer in Geographic Areas and Various Population Groups. Information is accumulating that certain forms of cancer in human population groups occur in a higher incidence than would be expected. Intensive study of such groups, and the recognition of possible etiologic agents which can be tested on experimental animals may identify some environmental factors which could be eliminated or controlled and thereby prevent many cases of human cancer. The following studies have this aim.

Maps have been prepared showing the mortality rates of 8 forms of cancer in the male, and 8 in the female in different geographic areas. Data were obtained from published reference material. A paper will be published in the J.N.C.I. in July 1968 (Dunham).

Bladder cancer in New Orleans. A total of 493 patients with histologically verified cancer, and 527 controls were interviewed, and environmental conditions were studied. A paper on results has been completed and submitted to the J.N.C.I. for publication (Dunham).

Uterine cervical cancer in New York City, Israel and Washington, D.C. This type of cancer was notably frequent in Negro women and infrequent in Jewish women. A report on the study in Jews and non-Jews in New York City and Israel was published in July 1966, a report on the Epidemiology of Tumours of the Uterine Cervix appeared in the European Journal of Cancer in March 1968, and a report on the study in Negro patients in New York City and

the Washington metropolitan area is being prepared. No statistically significant environmental differences have been found (Dunham).

A study of cancer in North American Indians has been completed. Cancers of the uterine cervix and the biliary tract were frequent in this ethnic group. Correlations with age and sex have been made. A review of the literature has been completed and revealed that very little reliable information is now available on the Indian population. Dr. Gert Laqueur now has the semifinal draft (Dunham).

Lung cancer in veterans of World War I. The medical history and biopsy and autopsy material from this group were gathered. A uniform classification of histologic types using the Kreyberg criteria is necessary for reliable comparisons.

No correlation between histologic type and cigarette smoking was found. Age at death from cancer was not related to age when started smoking, number of years smoked, or number of cigarettes per day. Not over 10% of heavy smokers had lung cancer. Epidermoid and oat cell carcinomas (not adenocarcinomas exclusively) were found in non-smokers. No increased risk was correlated with occupation. A publication has been submitted to Cancer. The same methods are being followed now in a study of lung cancer from La Plata, S.A. and Bristol, England (Herrold).

The number of cases of mesothelioma in South Africa and other geographic areas has increased surprisingly. It is suspected that exposure to asbestos is the cause. A mesothelioma panel has been organized, and criteria for histologic diagnosis will be established (Herrold).

Comparative pathology of gastric cancer in Japanese migrants. A review of the pathological material notes the location of cancer in the stomach, and any association with ulcer adenomatous polyps and intestinal metaplasia. This is a continuing collaborative study to include E.M. for virus particles and possible identification of cell of origin, and histochemistry (Herrold).

B. A natural extension of information on the epidemiology of cancer in man is experimental work with animals in a search for etiologic factors. Notable progress in the prevention of cancer has come from recognition of the carcinogenic activity of soot, shale-oil, and radioactivity. Once an environmental factor is suspected, tests on animals are necessary to prove that it is carcinogenic and in a complex substance it is important to identify the most potent fraction. A number of experiments with this aim are now in progress in the Laboratory of Pathology.

Betel quid chewing is associated with oral cancer in many areas of the world. Ingredients of the quid have been tested in the hamster cheek pouch. Calcium hydroxide was tested in combination with other factors, such as deficient diet, reduced salivary gland secretion, and intervening exposure to foreign proteins, silicate or plant materials. Epithelial atypia in the mucosa and inflammatory and hyperplastic lesions were found, but no cancer. An early report of this work has been published (Dunham).

A review of the literature on oral cancer and plants was presented at a symposium on carcinogens of plant origin. The effects of gambier, another ingredient of the betel quid, will be published (Dunham).

A study on multiple injections of adult rats with various fractions of heated fat and on the effects of feeding these fractions in the diet was begun by Dr. William Hueper. This has now been completed. A considerable number of sarcomas developed from multiple injections of adult rats with the non-urea adducts of corn oil and hydrogenated fats (O'Gara).

Esophageal cancer is high in Curacao, and natives use many decoctions of plants. Decoctions from 10 plants are being given orally to mice, or intramuscularly to rats. Only 1 sarcoma has developed in rats, but 8 in 23 mice had tumors, including several gastrointestinal ones, after mampuritu, 6 in 24 mice had lymphomatous neoplasms after kalbas. These substances will be further tested. No esophageal cancer has been found (O'Gara).

The possible carcinogenicity of fungi to man has been shown by the development of tumors after giving aflatoxin, a substance derived from contamination of peanuts by aspergillus flavus. Rats given aflatoxin B and G by stomach tube for long periods, showed no immediate toxic effects but later developed early hyperplastic nodules and small hepatomas in the liver and later hepatomas and carcinomas. One of these has been transplanted and proved to be a well-differentiated hepatoma. The results of this experiment are being prepared for publication, and a new experiment is planned using a non-alcoholic solvent. These findings may be relevant to the high incidence of liver cancer in parts of Africa (Snell).

The increasing number of lung cancers in man is a cause for concern. Asbestos is now suspected to be a carcinogen. No exact prototype for human lung cancer is known in animals, but a technique has been devised where a substance for testing can be incorporated in beeswax pellets and inserted into the lung tissue of a rat. The cancers induced by this method are epidermoid and histologically resemble human lung cancer.

After several years of effort, a more accurate method has thus been devised to determine the carcinogenicity for the rodent lung of substances in the human environment. Asbestos has been under investigation for more than two years with these results: (1) Beeswax pellets of asbestos alone - no tumors; (2) with MCA added - neoplasms, showing gradation from simple keratinized cysts to epidermoid cancer in over 70% of rats in less than 12 months; (3) Asbestos in gelatin saturated fibrous glass pads on pleural surface for 23 months - 75% of mesothelial sarcomas of different types - none in controls without asbestos. Experiments with fiber glass will be continued, and a possibility that newer types with a resin coating will be free of carcinogenic activity, and may even provide a protective mechanism. A basic experiment using the already developed techniques for lung cancer induction in rats is now in progress. DNA and RNA have been extracted from a previously induced transplantable epidermoid carcinoma from an inbred rat, suspended in the infarct, producing fluorocarbon and injected intravenously into rats of the same strain. It is postulated that molecular fractions from

the neoplastic cells may cause neoplastic alterations in stimulated normal cells of the same type. No results have yet been obtained, but this experiment takes advantage of a unique situation for testing a basic approach in carcinogenesis (Stanton).

The long term effects of an anti-fertility "pill" are under investigation. No effects were seen when Enovid was given in a powdered food. The Enovid was then administered in a liquid diet (Metrecal), and tested for nearly two years. The mice on Enovid were smaller and less fertile than mice on Metrecal alone, but no tumors have been found in the few autopsied. Mice on the liquid diet appear in good health, they have remained fertile for more than four generations, and no damage to the teeth has been noted (Dunn).

An axiom in cancer research is that many of the substances that are therapeutic are also cancerigenic. Examples are x-rays and nitrogen mustard. Yet almost none of the recently introduced chemotherapeutic drugs that are known to be radiomimetic and affect normal tissues in man have been tested for carcinogenicity in animals. Myleran and bisulfan are being given to hamsters in the drinking water and leukocyte counts and vaginal smears are followed. As yet, there are no abnormal findings (Chu).

Testing for modifications in carcinogenic activity that may be produced by other factors is an important area of research, but it requires many animals, and long continued observation. Experiments with hamsters show that Vitamin A will reduce the incidence of cancers in the forestomach in animals induced by chemical carcinogens, while alcohol seems to have the reverse effect. This work is continuing (Chu).

II. Cancer in Animals.

Many of the studies of cancer in animals are concerned with A) Induction and Pathogenesis, or the steps by which cancer develops after exposure to a known or suspected carcinogen; B) Data on various animal species and spontaneous and induced tumors in these species and C) Transplanted tumors.

A. The carcinogenic activity and mode of action of nitrosamine compounds is being studied. Nitrosamine compounds given by a variety of routes in hamsters produced tumors at similar sites, indicating that the distribution, metabolic pathways, and excretion of the carcinogen were the critical factors. Brain tumors have developed, raising the question whether nitrosamines can pass the bloodbrain barrier. Of special interest, there were malignant liver tumors arising from bile ducts and hemangioendothelial sarcomas, but no hepatic cell tumors. Odontogenic tumors and epidermoid cancers of the oral cavity have been induced. Pigmentary degeneration of the retina was found, and supplies an experimental model for this puzzling condition. These studies emphasize that carcinogens may reach remote sites, and that when the site of tumor formation is in the lung, the carcinogen did not necessarily reach the lung by inhalation. Gastrointestinal cancers developed when administration was by other than oral routes. These studies carried out by a pathologist are of special value because they furnish a review of all organ systems, and illustrate the complexity of a carcinogenic stimulus (Herrold).

Pulmonary lesions have been induced in hamsters by the intratracheal instillation of certain test substances, such as benzo(a)pyrene, cigarette smoke condensates, and thorium dioxide. Hormonal alterations were produced in some animals with nitrosamine injections. A description of fibrosing alveolitis and atypical proliferative lesions was published in the American Journal of Pathology in 1967 (Herrold).

Methylnitrosourea (MNU) was given by a number of routes to rats, which were then kept until moribund. The intragastric routes produced papillomas of the forestomach in all, and sarcoma of the glandular stomach in most. About one-half had tumors of the brain, spinal cord, mediastinum, uterus and mammary gland. By the intravenous route, tumors of brain, spinal cord, mammary gland and forestomach were produced. By the subcutaneous route, tumors were induced at the injection site, and in females a few tumors of the mammary gland and uterus. These experiments are important because they emphasize further the widespread effect of some chemical carcinogens, and they give warning of the danger of proposed commercial use of MNU (Snell).

Studies on the induction and histopathological analysis of transplantable bone tumors produced in rats by cupric-chelated N-OH-acetylaminofluorine is continuing. The morphology of the tumors is altered during transplantation - some produce bone, some cartilage, and some possibly blood forming elements. These tumors closely resemble osteogenic tumors of man and may provide a clue to the variable behavior of human tumors of this type. The tumors are being stored in liquid nitrogen and have been provided to outside investigators. EM has not so far revealed any virus particles, but more work was indicated when EM facilities are available. Factors influencing differentiation will be studied further (Stanton).

The pathogenesis of tumors induced by metals is being studied. When smooth or rough surfaced iron disks were inserted into the subcutaneous tissues of rats, fibrosarcomas developed in both groups, but were earlier in the smooth. The reaction to copper disks inserted deep in the muscles was remarkable. Fluid filled cysts developed and persisted; in 8-11 months, 3 sarcomas have been found in the walls of these cysts. Aluminum disks produced tumors in 8/18 rats, and zinc caused 1/25. If metal disks become corroded, and have a rough surface, yield of tumors is less. Plastic disks have now been inserted, and MCA given orally. No further work will be done until the effect of plastic disks is determined. Importance of this work is the present use of metals and plastics in surgery, and accidental incorporation in human tissues (O'Gara).

Although the carcinogenicity of many chemicals is easily demonstrated, it is important to know how the activity can be modified by different environmental conditions or diets. This is especially important in relation to cancer in man where a weak carcinogen may be enhanced and multiple factors are involved. The possibility that carcinogens may be additive in their effects should not be overlooked. A number of experiments are now in progress to disclose modifying factors: (1) Riboflavin analogues enhanced the effect of azo-dye hepatocarcinogenesis. A report of this has been submitted for publication (Mulay). (2) Small doses of extracts of fatty acids and fats

irradiated with ultraviolet light enhanced the effect of hepatocarcinogens, but higher doses of these UV-irradiated extracts were too toxic for long continued work (Mulay). (3) Higher doses of Me-DAB were required for carcinogenesis of rats on a Purina chow diet, than for rats on a semi-synthetic diet. A dose response curve has been established, which is a necessary preliminary for further work (Mulay). (4) DENA and DAB are more effective hepatocarcinogens if given in combination than if given alone (Mulay).

Attempts are being made to induce transplantable lung tumors in rats by the subcutaneous and intragastric administration of nitroquinoline, and kidney tumors by nitrosamine. No success has yet been achieved. Attempts to propagate lung tumors with cell free nucleic acid preparations were done in collaboration with Dr. Stanton (Mulay).

B. Data on various animal species and spontaneous and induced tumors in these species.

Because the training of pathologists is not restricted to one organ system or type of disease, they are often able to contribute to the general knowledge of the normal and pathologic anatomy of many different animal species. The incidence and types of cancer are recorded, and any alterations in the tumor bearing hosts, or systemic effects of the tumors are noted. This contribution is indispensable to the intelligent use of biologic material, because, unless the normal anatomy and spontaneous pathologic alterations are recognized, it is impossible to interpret the effects of experimental procedures. The size of the staff has made it possible for various members to become specialists in a particular species.

A review paper based on publications and personal experience on the "Hodgkin's like lesion" in the mouse will appear in the April issue of J.N.C.I. This lesion is frequent in old mice, and has some resemblance to Hodgkin's disease in man. Work is in progress on a review paper on the adrenal gland of the mouse. Dr. Victor Swaen, a guest worker from the Netherlands, has studied much of the material accumulated here. Special studies have been made on the pathogenesis of adrenal cortical tumors. Papers on Amyloidosis, and on Renal Disease in Mice were published this year. A group of old untreated BALB/c mice has been autopsied, and various tissues transplanted. An inbred line of wild mice developed by Dr. Andervont, and hybrids of these mice with high and low amyloid strains is under study (Dunn).

Studies on 6 inbred strains of rats and one germ-free group are continuing as old rats live out life span. No new data has been added, but a reliable base line for the expected incidence of tumors and other lesions is being established. A review on renal disease in rats was published during the year (Stewart-Snell).

Mastomys provide a recently introduced laboratory animal that have given an opportunity for the study of cancer and degenerative disease in a different species. Recent studies on spontaneous tumors found in old Mastomys have been directed toward neoplasms of the reticuloendothelial system, and thymomas which are often accompanied by myositis. This provides a parallel to myasthenia gravis in man. Histologic sections on 200 old animals have

been made, serial killings are being done to watch the evolution of the thymic lesions, and collaborative work on enzymes and autoantibodies is in progress. The possibility that the gastric cancers found in Mastomys are carcinoid is being investigated. A paper on hematopoietic diseases is in preparation, and a paper on thymomas is in press (Stewart-Snell).

Primates are being more widely used, and special centers for their study have been set up. Information acquired in the Laboratory of Pathology is important. Monkeys injected more than 16 years ago with MCA into the wall of the stomach are still under observation. No gastric cancer has been found, but these animals will provide valuable information on the pathologic anatomy of old primates. Two cases of spinal ankylosis, one of which resembles Marie-Strumpell disease in man has been described in collaboration with Dr. Sokoloff. One case of bilateral renal carcinoma has been found (Stewart-Snell).

The effect of carcinogens on newborn and immature primates is being studied, and basic information is being obtained on younger animals. A breeding colony with about 150 adult females and 80 live births per year is maintained. One hundred eleven autopsies have been performed, 18 given DENA have been autopsied and all had hepatic cell carcinomas. In 22 biopsies, 18 had hepatomas. One monkey receiving nitrosopiperidine had a large hepatoma. Other carcinogens being tested are aflatoxin, azo-dyes, and benzpyrene. The finding of alpha-fetoprotein in the monkeys with hepatic cancer is especially important, since it has been found in human cases, and it can be used as a test for the presence of cancer and the effectiveness of treatment. In the primate experiment, a correlation with histopathology and liver function tests can be made. It is emphasized that experiments with primates require many years to complete, often 10 years or more (O'Gara).

B. Invertebrates and lower vertebrates, phylogenetic aspects of neoplasia.

This important project is continuing in cooperation with the Smithsonian Institution and marine biological laboratories. Dr. J. Harshbarger, working at the Smithsonian, is in charge of this project, and the number of accessions has increased from 76 to 123. Since this is a new field of research, it is necessary to establish criteria for the identification of neoplasms, since there has been much confusion in distinguishing neoplastic, hyperplastic and regenerative responses. In order to establish these criteria more firmly, cockroaches are now maintained in the laboratory, and injected with tissue culture cells from vertebrates. The response resembles the immune cellular response of higher animals and shows that lower animals have an effective policing system against heterologous tissues. The mechanism by which lower animals protect themselves from neoplasia will be studied. The collection of publications relating to neoplasms in lower animals is continuing. An

important undertaking is the organization of a Symposium to be held June 19-21, 1968 at the Smithsonian since this is the first gathering devoted to this subject.

C. Transplanted tumors. Observations on transplanted tumors and their behavior furnish valuable information on neoplasia. A number of these are carried and studied in the Laboratory of Pathology, and are made available to other investigators.

Mouse. About 20 tumors are being carried in the mouse. Of particular interest are a myoepithelioma that produces granulocytosis, a renal tumor, and lymphoid tumors resembling Hodgkin's disease in man. Transplantable adenocarcinomas of the uterus were studied by Dr. Nubia Munoz, a visiting scientist from Colombia, S.A. One of these proved to be hormone dependent, and grew better under estrogen stimulation (Dunn).

Rat. A number of tumors are being carried in the rat. Of special interest are a hormone dependent adrenal cortical tumor, a gastric adenocarcinoma, and a rhabdomyosarcoma (Stewart-Snell).

Thirteen tumors of different morphological types derived from bone are being carried (Stanton).

Hamster. A contagious tumor in the hamster has been under study for several years and the mode of transfer is being investigated. It was proved that it could be transmitted by feeding tumor tissue to susceptible hamsters and it has now been shown that it can be transmitted by the bite of the Aedes aegypti mosquito. Transmission is by transfer of cells. Several other species of mosquito failed to transmit it, and two other hamster tumors and the Shope fibroma were not transmitted. This mode of transfer may be possible in human tumors, and it also raises the consideration of the consequences of the transfer of incompatible cells and proteins. Work is being planned with an epidemiologist to collaborate on an epidemiologic approach to cell transmission in neoplasms. A research fellow, Dr. Masaki Ohmori, has obtained excellent electron microscope photographs of the Aedes mosquito and will compile an atlas of the normal adult. He has also discovered a new type of striated fiber and a double membrane vesicular structure in the development of Shope fibroma virus, and a doughnut shaped structure in the early developmental forms of the elementary body (Banfield).

Primates. A transplantable hepatoma in a primate is now being studied. The tumor was induced by DENA and the transplants are made intracerebrally from cells grown in tissue cultures into juvenile or adult monkeys. Immunosuppressive drugs were used, and the transplants showed progressive growth. This tumor will be especially useful in testing chemotherapeutic drugs, and in the recognition of alpha-fetoprotein, a substance that may help in the

diagnosis of liver cancer in man, and in following the clinical course and response to chemotherapy (O'Gara).

III. Studies at the Cellular or Molecular Level.

During the past ten years, experimental work in cancer has concentrated on the cell. This is a natural extension of work on the total animal, and it is made possible by the development of new techniques where the single cell, and structures within that cell, can be closely examined. Many members of the Laboratory of Pathology have become expert in these exacting new methods, and their broad background in medicine and pathology gives them a breadth of understanding of the total problem that makes their contributions especially valuable. These techniques include cytogenetics, tissue culture, immunocytology, electron microscopy and virology.

A. The cytogenetic characteristics of long term human lymphoma cells in vitro, and patients with premature ovarian failure are under study. Some of the lymphoma cells were infected with a Simian virus. Chromosome analysis revealed that a marker chromosome disappeared in one line, but persisted in another. One patient with premature ovarian failure had 47 chromosomes with trisomy X, and this abnormality may be significantly related to her condition. This work will continue, and be extended to studies on primary and metastatic tumors (Chu).

B. A tissue culture study of a liver tumor in a rhesus monkey induced by Dr. Kelly and Dr. O'Gara with nitrosamine is continuing. Most tissue culture lines are epithelioid (E) but fibroblast-like variants have developed (F). These lines have developed distinctive karyotypes and show chemical and morphological differences. One E subline contains alpha-fetoglobulin, an antigen found in murine and human liver cancer; this substance was not found in the F subline. These tissue culture lines are useful for metabolic studies, for virus propagation to study hepatitis, and for alpha-fetoglobulin determinations. The ability to transfer cells back to the primate offers special opportunities for study (Dawe).

C. The field of immunocytology is especially promising in cancer research today, and several studies are being carried out in the Laboratory of Pathology.

A number of techniques are being adapted or originated and applied. The fluorescent antibody technique has demonstrated a surface antigen on polyoma transformed mouse and hamster cells which reacted with mouse anti-polyoma virus and antipolyoma tumor sera. Normal mouse sera, or sera from mice immunized with other virus tumor cells failed to react. Similarly, non-transformed mouse cells or SV40 virus transformed mouse and hamster

cells failed to react. The surface antigen was also demonstrated on cells in the lytic cycle (Malmgren).

Normal and cancerous human sera are being surveyed for adenovirus 12"T" antibody. Sera from both sources reacted, but cancers of the breast, brain and colon were most frequent. This was not considered proof, however, that neoplasms of this type were caused by adenovirus 12. Anti-melanoma antibody is being looked for in the serum of normal and of melanoma patients. Antibodies were found in 7 of 27 patients against their own melanomas. This points to an immunologic response which indicates that tumor is antigenic. Possible viral nature of the antigen was suggested by similar antibody in melanoma patients as well as normal individuals (Malmgren).

Immunologic and other factors involved in carcinogenesis are being studied. Evaluation of methods for separation of solid tumors into single cells has been made and it has been found that the proteolytic enzyme Pronase provides the greatest number of viable cells per unit weight of tumor with the least cell debris of the various methods tested (Malmgren).

Moloney virus infection was enhanced by DNA and RNA obtained from a commercial source (Malmgren).

Mechanisms of action in tumor immunology are poorly understood, and require much more work. The relative importance of cellular and serologic factors especially needs study. Fluorescence microscopy was used, and it was found that (1) There is no serologic immunity in chemically induced tumors, but "T" antigen was found in SV40 tumors. (2) Cellular immunity is being studied by a newly developed in vitro method. Viable, rapidly dividing, single cell suspensions of an MCA induced fibrosarcoma were tested, and found to be more affected by peritoneal cells from an immunized, in comparison with a control animal. Growth inhibition appears to be a primary effect, but this is difficult to demonstrate in a syngeneic system. Using this technique, studies on virus induced and human tumors will be undertaken.

D. Electron microscopy.

Technical advances have been made with the electron probe, an instrument which permits identification of elements within a cell. The scanning electron microscope resolution has been greatly improved as has the x-ray analytical resolution and the biologic application of this new instrument is being studied. A contract with Biodynamics Research Corporation is being continued. Rapid progress has made it feasible to undertake such studies as (1) freeze drying for elemental analysis of skin and thyroid gland, the latter especially for iodine, (2) analysis of asbestos bodies, (3) analysis for titanium in lungs, (4) analysis of electrolytes, calcium and

potassium in cartilage and, (5) a study of intracellular calcification. It is proposed that an electron probe laboratory be set up intramurally within the National Institutes of Health so that a source of experience for the incorporation of this instrument into the programs of the Institutes can be obtained and so that development of the instrument for biological application can be facilitated. The electron probe should prove to be of great value in medical research, since the in situ analysis of tissue specimens localized to a μ^3 area can be accomplished by no other instrument. It will undoubtedly play a part in the investigation of the effects of the increasing exposure of human populations to extrinsic substances which may be disease hazards (Banfield).

Since cancer is a pathologic form of growth and differentiation, it is appropriate that a rapidly growing, differentiating normal tissue that is hormonally responsive should be studied by EM in order to identify the morphologic changes in a normal growth process. Study of the immature chick oviduct has shown (1) A homogenous epithelium under estrogen stimulation develops into 3 distinct cell types (2) Albumin is formed in tubular gland cells (3) Estrogen induces specialized goblet cells leading to avidin formation, and prolonged estrogen stimulation produces a granule and ovimucin (Grimley).

An EM study on collagenous connective tissue and elastin is continuing. A paper describing an elastofibroma has been completed, and is one of the first studies on collagen pathology at the EM level. It has been found that formalin fixed tissue can be used even after long storage. Future studies will be concerned with (1) age changes in human skin (2) phylogenetic development of elastin (3) degeneration of collagen in mastocytomas of dogs and (4) changes in elastic fibers in mycosis fungoides (Banfield).

Neuroendocrine tumors in man are being studied. The EM features will be correlated with evidence of hormone production in order to obtain an experimental system for the study of neurohormone synthesis. These tumors appear to produce catecholamines, which suggests a common ancestry. Unique cytoplasmic granules provide a persistent marker for observations of the neoplastic change (Grimley).

EM study of human tumors is continuing. Tumor cells in vitro are compared with original cells from surgical specimens, and viral particles are searched for. Improved techniques make this type of study more profitable, and medullary carcinoma of the thyroid, Ewing's sarcoma and malignant lymphoma are under study (Grimley).

E. Ultrastructural Research on Viruses. The EM is now an indispensable instrument for the observation of viruses, and a number of studies are in progress.

Epizootic diarrhea in mice is caused by a virus. This has not grown in the usual tissue culture, but efforts will be made to grow it in organ culture. The virus shows many developmental forms and is an ideal subject for complete ultrastructural histology of a virus life cycle. Morphologically it is unlike other RNA viruses (Banfield).

A small dense granule has been seen in the elementary body of molluscum contagiosum and also in the Shope fibroma elementary body. Histochemical methods are being used and the nature of this granule is being investigated (Banfield).

Ultrastructural studies in arbovirus infection will be correlated with biochemical studies during arbovirus synthesis. Unique structures thought to represent centers of viral synthesis in chicken fibroblasts have been demonstrated, and viral entry and release have been observed. Progressive changes after virus infection will be looked for (Grimley).

Herpes simplex infection is being studied with the EM by Dr. Victor Bedoya, visiting fellow. When cell lines are developed from patients with malignant lymphoma, intranuclear "herpes-like" particles usually appear, and morphologic features will be observed. Other investigations will be the growth cycle of herpes simplex in infected cells, the EM and cytofunctional alterations in virus infected cells, and conditions altering host cell susceptibility. It has been found that the herpes simplex virus infection is similar to that which occurs spontaneously and leads to disfigurement of the nuclear envelope and formation of Type A intranuclear inclusions (Grimley and Rabson).

F. Polyoma virus tumors in mice - interrelation of epithelium and mesenchyme. Since this research has been in progress for 8 years or more, a review of some of the previous work is required. The fundamental problem of the interaction of epithelial and mesenchymal elements is being continued, and tumors produced by the polyoma virus are utilized for this study. The results have extended beyond a simple consideration of a virus-host relationship. The specificity of the mesenchyme for the epithelium is critical, and some combinations produce no neoplastic change, even in the presence of polyoma virus. Continued contact with natural mesenchymal tissue is required for epithelial neoplasia, and neoplasia will not develop until some morphogenesis and differentiation take place. The salivary gland rudiments must reach a certain stage before neoplasia results because pre-rudimentary tissue did not produce tumors. If epitheliomesenchymal relationship was disrupted, no tumors developed, but when recombined, tumors developed. The salivary gland epithelium required its own mesenchyme for growth.

Now that these facts have been established, the recent progress can be summarized. (1) Rudimentary salivary gland epithelium placed in growing polyoma induced salivary gland tumor tissue and allowed to remain for 12

days showed morphogenesis and differentiation. The same methods were used with other rudiments, and it was found that morphogenesis occurred with salivary and pancreatic epithelium, but not with odontogenic and lung epithelium. Fibrosarcomas induced by PV, and myoepitheliomas did not support morphogenesis, so thus far only PV induced salivary gland tumors among all the combinations tried have this power to support morphogenesis; (2) The PV salivary gland tumors in mice contain both epithelial and mesenchymal elements, and it is uncertain which is the essential neoplastic element. T6 chromosome from CBA/T6 mice markers are now being used to test these elements, but no results have been obtained; (3) EM study of normal salivary gland in PV tumors is being made in an attempt to visualize the border between normal and tumor cells. No definite results have yet been obtained; (4) Time lapse phase studies are in progress to observe the interaction between PV tumor and normal cells. Salivary epithelium in a PV tumor grows over the external surface, while normal epithelium in a normal mesenchyme forms within a mesenchymal capsule. The possibility that neoplastic epithelium may become independent of mesenchyme will be studied; (5) Work has just started to see whether a subcellular substance will pass through a millipore filter, and alter morphogenesis or neoplasia (Dawe).

G. Viral carcinogenesis.

SV40 and oncogenic human viruses in vitro and in vivo are being studied in Syrian hamsters. Much of the work is collaborative. The major findings are (1) Transformed lines derived from the hamster prostate have contained "prostatic" acid phosphatase; (2) Explant cultures of adult hamster ocular tissues were infected with four oncogenic DNA viruses. Most cultures underwent transformation, and produced tumors composed of epithelioid and spindle cells. When injected into irradiated hamsters, no pigmented tumors were found; (3) Transformed lines of pineal glands of monkeys are being studied for enzyme content and ability to produce tumors when injected; (4) Transformed cells from a human parathyroid adenoma have produced parathyroid hormone; (5) That the adenovirus 12 genome may determine the morphology of the resulting tumors is indicated by their remarkable uniformity. The results of these experiments indicate that virus altered cells may still show hormone function and that the virus genome is incorporated into the cell (Rabson).

The search continues for a virus in a human lymphoma, and the biological properties of human malignant lymphoma cells in vitro are being studied. Another line of lymphoblasts has been isolated from an American patient with Burkitt lymphoma, and it has a herpes virus that is not significantly different from that found in other lines. The growth of the herpes simplex virus in lymphoma cells is being studied by Dr. Victor Bedoya, a research fellow.

Cytomegaloviruses are also being studied, with the possibility that they may be involved in human lymphoma. Attempts have been made by Dr. Mary Ann Guggenheim, a postdoctoral fellow, to recover a latent viral genome from human lymphoma cells (Rabson).

Cervical lesions in hamsters and the action of herpes virus is being studied. If hamsters are immunized against herpes virus, and then the cervix is painted with podophyllin, a severe cervicitis results, but no neoplasms as yet. If 10% alcohol and podophyllin cervical painting is made, and herpes virus given, a severe dysplasia results but no neoplasms (Chu).

The murine sarcoma virus (MSV) has been intensively studied by virologists, but very little work has been done on the morphology of the tumors it induces. Infected mice have now been killed and autopsied at frequent intervals, and the resulting lesions studied. The response is unlike that of any previously studied oncogenic virus. Early changes are mostly granulomatous, but they contain a smattering of cells not typical of inflammation. The findings can be summarized (1) Initial inflammatory tissue, but containing some atypical cells not found in ordinary inflammatory tissue. (2) A small percentage of the lesions develop into simple mesenchymal neoplasms, but one tumor was a mammary tumor of unusual morphology. (3) Unique reactive response resembles some complex human neoplasms, such as Kaposi's sarcoma, Hodgkin's disease, or reticuloendothelioma. (4) EM shows unique alteration in cell organelles, regardless of the cell type that responds and some of these EM characteristics are also reported in the avian-leukosis-sarcoma complex (Stanton).

H. Basic virus research and interferon.

Replicative forms of an RNA virus are being studied by viral plaque assays, RNA extraction, sucrose gradients, scintillation counting, E.M., base ratio analysis, lipid analysis and column chromatography. Four replicative RNA forms of an arbovirus have been found and characterized. The formation can be blocked by interferon, but not by actinomycin D. All four forms are associated with distinct cytoplasmic particles. The protein synthesis by the virus is carried out by nascent RNA that has not yet disengaged from the template. Studies of this RNA virus may be applied later to studies of a tumor virus (Friedman).

The production of interferon by non-viral substances, especially human mitogens is under study. Cultures of human and mouse cells are used. Results show that mitogens in human lymphocyte cell cultures also induce interferon production. Stimulation of DNA synthesis is produced in other systems. Therefore, interferon may be a concomitant of cell growth (Friedman).

Another study on interferon concerns its possible role in viral oncogenesis. Standard methods for virus and interferon assay are used. It was found that interferon action is a 2-step process (1) attachment of interferon to cells at 1°C. The conclusion reached was that interferon appears to be exclusively an inhibitor of viral RNA production and it may be useful in controlling oncogenic viruses. This work may also indicate a difference between cell and viral RNA, and in pinpointing sites of cellular RNA control mechanisms. An attempt will be made to show a direct effect of interferon on viral protein synthesis (Friedman).

VI. Collaborative Research.

It is recognized that many research projects at the National Cancer Institute require the collaboration of a pathologist, especially in the final evaluation of the effect of an experimental procedure on laboratory animals. The Laboratory of Pathology has always tried to make this assistance available.

A. Methods. The pathologist may take an active part in planning an experiment and in following it through; he may take the responsibility for all autopsies and histologic diagnoses in an experiment; he may review only the histologic sections in a given experiment; or he may serve as a consultant to review selected material with no responsibility for the entire experiment and its publication. Finally, he may make use of material accumulated by other investigators for independent studies concerning pathologic alterations. It is emphasized that full collaboration of the pathologist at the time the experiment is planned is the most satisfactory arrangement for it insures the best and most economical selection of material for pathologic studies.

In addition to the use of the light microscope and standard autopsy procedures, individual members of the Laboratory of Pathology have become proficient in special techniques such as fluorescent antibody visualization, electron microscopy, tissue culture, autoradiography, exfoliative cytology, special cytology, and histochemistry. These special skills are often made available in collaborative studies.

B. Examples. It would be tedious to consider all the collaborative work now in progress in the Laboratory and this should be unnecessary since it is covered in reports from other laboratories. However, the following are noteworthy.

Anterior chamber transplants of tissue culture cells are examined for tumor growth, and for any alterations to eye structure caused by the procedure (Dunn).

Gonadotrophic hormone has been used to induce ovulation in women who have been sterile. Pregnancy has followed in 13 patients. The information from this study may help to induce or to prevent pregnancy (Powell).

Experimental emphysema has been induced in rabbits by elevation of intratracheal air pressure, in an attempt to reproduce the human disease. The pulmonary injury in the rabbits heals spontaneously and they become resistant (Powell).

Other collaborative work with clinicians will appear in the report of Dr. Louis B. Thomas.

V. Examples of Special Contributions and Recognition to Members of Staff.

A. Editorial:

Scientific Editor, JOURNAL OF THE NATIONAL CANCER INSTITUTE: Dr. Stanton

Associate Editor, INTERNATIONAL JOURNAL OF CANCER: Dr. Dawe

Chairman, Editorial Board, Tissue Culture Association: Dr. Dawe

Founding Editor, IN VITRO, TCA Annual Monograph: Dr. Dawe

Editorial Advisory Board, CANCER RESEARCH: Dr. Dunn

Associate Editor, JOURNAL OF THE NATIONAL CANCER INSTITUTE: Dr. Malmgren

B. Officials in Organizations:

President, Washington Society of Pathologists: Dr. Edgcomb

President, NCI Assembly of Scientists: Dr. Edgcomb

Executive Committee, American Society of Cytology: Dr. Malmgren

Executive Committee, Joint Committee on Clinical Stage Classification and End Results Reporting: Dr. Thomas

Assemblyman, Maryland Society of Pathologists: Dr. Thomas

Past President and Member of Executive Committee, International Council of Societies of Pathology: Dr. Stewart

Executive Committee and Council, UICC: Dr. Stewart

Chairman, Committee on Manual of Tumor Nomenclature & Coding (Revision of Red Book), Publication of 1968 Edition in July 1968: Dr. Thomas

C. Participation in Meetings and Symposia:

Electron Microscopy Society of America, Chicago, August 1967. "Further Observations on the Virus of Epizootic Diarrhea in Infant Mice. An Electron Microscope Study." Dr. Banfield

Symposium on Electron Probe Analysis in Biology and Medicine, New York, November 1967. "Electron Probe Detection and Localization of Titanium in Human Lung Tissue": Dr. Banfield

Symposium on Comparative Morphology of Hematopoietic Neoplasms, AFIP, Washington, D.C., March 1968: Drs. Stewart, Dunn, Snell, O'Gara, Banfield, Dawe, Berard, Thomas

Symposium on Cell Culture of Virus Vaccine Production, Bethesda, November 1967: Drs. Dunn, Rabson, Dawe

Symposium on Carcinogens of Plant Origin, Bethesda, April 1968: Drs. Stewart, O'Gara, Dunham

Conference on Fuller Utilization of the Woman Physician. Washington, D.C. January 1968: Drs. Dunn, Todd

Staige-Blackford Memorial Lecture, University of Virginia, Charlottesville, Va., December 1967: Dr. Dunn

Committee on International Congresses of the International Union Against Cancer, Geneva, Switzerland, May 1968: Dr. Stewart

World Health Organization Meeting of Heads of International Reference Centers for Pathological Definitions of Types of Cancer, Geneva, Switzerland, December 1967: Dr. Stewart

Committee to Advance Worldwide Fight Against Cancer, New York, January 1967: Dr. Stewart

M.D. Anderson Hospital, Houston, Texas, September 1967, "Problems Related to Cancer in Man": Dr. Stewart

Organizing Committee on International Congresses for Tenth International Cancer Congress in 1970, Dallas, Texas, May 1967: Dr. Stewart

WHO Meeting of Investigators on Histopathological Nomenclature & Classification of Uterine Tumours, Geneva, Switzerland, November 1967. Dr. Thomas

Conference of Investigators on the Histopathology of Burkitt's Tumor, October 1967: Dr. Thomas, Dr. Berard, Washington, D.C.

Symposium on Pathology of the Larynx, AFIP, Washington, D.C., March 1968: Dr. Thomas

U.S.-Japanese-Symposium on Malignant Lymphomas, Bethesda, November 1967, Drs. Thomas, Berard

D. Services on Committees, Contracts, Panels:

Contract - Biodynamics Research Corporation, Inc. (Electron Probe): Dr. Banfield

Contract - Melpar, Inc., (Insect Transmission): Dr. Banfield

NCI Chemical Carcinogenesis Contract Review Committee: Dr. O'Gara

Contract - Smithsonian Institution (Registry of Tumors of Lower Animals): Drs. Dawe, Stanton

Committee on Special Animal Leukemia Ecology: Drs. Stanton, Dawe

NCI, GL&C Contract Program Review Board, Exec. Secty: Dr. Rabson

Special Virus Leukemia Program. Development and Research Segment: Dr. Rabson

Contract - Baker Histology Laboratory (Slide Preparation): Mr. Albrecht

Mesothelioma Panel: Drs. Stewart, Herrold

Advisory Cancer Council, NCI: Panels on Chemical Carcinogenesis: Drs. Stewart, Dunn, Stanton, Dawe, Snell, O'Gara, Thomas

Committee to Organize Symposium on Tumors in Invertebrates: Dr. Dawe

Lymphoma Task Force: Drs. Thomas, Berard

America Joint Committee for Staging and End Results Reporting of Hodgkin's Disease: Dr. Thomas (Chairman), Dr. Berard

Budget Committee, International Academy of Pathology: Dr. Thomas

Biometry & Epidemiology Contract Review Committee: Dr. Thomas

International Reference Centre for Histopathology of Uterine Tumors and Related Condition, UICC: Dr. Thomas

Committee to Advance the Worldwide Fight Against Cancer, ACS: Dr. Stewart

Clinical Professor of Pathology, Georgetown University: Dr. Stewart

Consultant, Doctors Hospital, Washington: Dr. Stewart

Special Virus Leukemia Program; Testing and Monitoring Committee: Dr. Malmgren: Contract - Immunofluorescence Studies of Lymphoma and Leukemia (The Children's Hospital of Philadelphia); Contract - Human Leukemia Virus Test System Problem Survey (Wistar Institute)

NCI Diagnostic Research Committee: Dr. Malmgren

Serial No. NCI 500
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Histogenesis and Pathology of Induced and Spontaneous Tumors of Laboratory Animals

Previous Serial Number: Same

Principal Investigator: Harold L. Stewart

Other Investigators: Katharine C. Snell

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 4
Professional: 4
Other: 0

Project Description:

A. Untreated Mastomys

Objectives: To study and describe the tumors and other lesions that develop spontaneously in Mastomys. During the past year, the emphasis has been on thymomas and thymic hyperplasia (with concomitant myositis, myocarditis, and sialodacryoadenitis) and on neoplasms of the hematopoietic system.

Methods: (1) Histologic sections from over 200 old Mastomys are being reviewed in order to diagnose, tabulate and describe the hematopoietic neoplasms. (2) The same procedure has been completed on the thymic lesions. In view of the major findings (see below), serial killing of groups of Mastomys from birth to old age is underway in order to study the involution of the thymus and the evolution of thymic hyperplasia and thymomas. (3) A collaborative project with Dr. Arthur Strauss (NIAID) on the possible relationship of thymic lesions to myasthenia gravis in man has been begun.

Major Findings: 1. Hematopoietic neoplasms have been diagnosed in approximately 10% of Mastomys necropsied. Reticulum cell sarcoma is the most frequent of these neoplasms, but several cases of lymphosarcoma and one case of plasma cell myeloma have been observed. A summary of these findings was presented at a symposium on comparative morphology of hematopoietic neoplasms at the Armed Forces Institute of Pathology on March 11, 1968.

2. A paper on thymomas and thymic hyperplasia is in press. Twenty seven thymomas and 11 hyperplastic thymuses were observed among 213 Mastomys necropsied. Polymyositis was observed in 1 Mastomys with thymic hyperplasia and in 7 with thymomas. Myocarditis was present in about 1/3 and sialodacryoadenitis was present in about 2/3 of the animals with thymomas.
3. In the collaborative project with Dr. Arthur Strauss on the thymus of Mastomys and the possible relationship of thymic lesions to myasthenia gravis, immunoglobulins from Mastomys' serum have been prepared for anti-Mastomys IgG serum-fluorescent conjugate. Autoantibodies to striated muscle have been identified in a Mastomys with a thymoma and polymyositis. (Such antibodies have been demonstrated in patients with thymomas and myasthenia gravis and/or polymyositis). A control series of pooled and individual sera from Mastomys without thymomas did not show similar antibody reactivity. A preliminary report on this finding is being prepared for publication. This collaborative project will be more completely described by Dr. Strauss in his annual report.

Significance: Mastomys are becoming of increasing importance as experimental animals since in many ways their diseases parallel those of man (cancer of the stomach, osteoarthritis, glomerulonephritis, for example). If the relationship of thymomas, polymyositis and autoantibodies to striated muscle can be further elucidated, Mastomys might ultimately serve as a useful spontaneous model for the study of the chronologic and functional relationships between thymoma and anti-striated muscle autoantibodies, and possibly polymyositis and myasthenia gravis. The continued study of untreated Mastomys is important in order to establish a baseline for future experimental work.

Proposed Course: Complete a paper on hematopoietic diseases; complete the preliminary report and continue to study the thymomas, polymyositis, and serum autoantibodies to striated muscle in Mastomys; investigate the possibility that the cancer of the glandular stomach is in reality a carcinoid tumor; investigate the serum and urinary proteins of Mastomys in relationship to their renal disease.

B. Monkeys

Objectives: Originally the objective of this experiment was to determine whether 3-methylcholanthrene injected into the wall of the stomach would induce gastric carcinoma. Since, after many years of observation, no gastric cancers have been found, we are keeping the remaining monkeys to study any tumors or other lesions that may occur with age.

Methods: 3-methylcholanthrene was injected or implanted in the wall of the stomach of Rhesus monkeys 16 to 18 years ago. The monkeys, which are now 18 to 20 years old are being observed and allowed to live as long as possible.

Major Findings: Nothing significant has been observed in the stomach of any monkey necropsied. Recently, however, two cases of spontaneous spinal ankylosis have occurred and been studied in collaboration with Dr. Leon Sokoloff (NIAMD). One case closely resembles Marie-Strümpell disease, a condition not known to occur in species other than man; the other case was interpreted as hyperostotic spondylosis. This second animal also had bilateral carcinoma of the kidney.

Significance: With the increasing use of monkeys in cancer research it is important to study old animals to determine the incidence of spontaneous disease. Very few colonies of monkeys of known and advanced age are in existence so that our animals constitute an important group for future study. Hyperostotic spondylosis is a disorder that is currently the subject of considerable clinical study. The finding of this and of Marie-Strümpell disease in these monkeys suggests that they may offer an opportunity for observing changes in bone which are almost never available in human autopsy material.

Proposed Course: Continued observation of monkeys with necropsies as necessary. Completion of a paper entitled: Spinal ankylosis in old Rhesus monkeys.

C. Untreated Rats

Objectives: To determine the incidence of tumors and other lesions that occur spontaneously in rats of 6 inbred strains and in a group of germ-free rats.

Methods: In the early part of this experiment rats were serially killed at 3-month intervals. That part has been completed and we are now adding data on rats allowed to live as long as possible as well as on old germ-free rats.

Major Findings: None other than reported for 1966-67.

Significance: The knowledge gained from a study of neoplasms and other lesions that occur spontaneously in laboratory animals can provide a basis and possible analogy to the study of the tumors and other lesions that occur in human beings.

Proposed Course: Continuation of the study of untreated and germ-free rats as old animals become available.

D. Carcinogenicity of Methylnitrosourea (MNU)

Objectives: To determine the site of tumor induction and the pathology of the tumors induced when MNU is administered to rats by various routes.

Methods: MNU in aqueous solution was administered to male and female Buffalo rats intragastrically by stomach tube, intravenously, and subcutaneously for 20 weeks. Total doses amounting to between 50 and 100 mg. per

100 grams of rat were given to each animal by each route. The rats were allowed to live as long as possible and killed when moribund; in most cases this was between 300 and 400 days after the beginning of treatment.

Major Findings:

1. Intragastric administration of MNU induced papillomas of the forestomach in 100% of animals and sarcomas of the glandular stomach in nearly that number. About 1/2 of the rats developed tumors of the brain or spinal cord; tumors of the mediastinum, uterus, and mammary gland also occurred.

2. Intravenous administration of MNU induced tumors of the brain, spinal cord, mammary gland, and forestomach (papillomas) were induced.

3. Subcutaneous administration of MNU induced tumors of the injection site in all and in females and a few tumors of the mammary gland and uterus.

Significance: MNU and related compounds are used or have been proposed for use as solvents, high pressure lubricants, insecticides, and as stabilizers in the synthetic rubber industry. The investigation of tumors induced by such compounds in animals may be highly important for the human population.

Proposed Course: Study and evaluation of histologic sections followed by preparation for publication of the results obtained.

E. Carcinogenicity of Aflatoxin

Objectives: To study the carcinogenicity of aflatoxin for the liver and other organs of the rat.

Methods: Aflatoxin B_1 and G_1 as a mixture was obtained from the Toxicology Unit of the Medical Research Council of England. An alcoholic solution was prepared and administered to Buffalo rats by stomach tube so that each rat received 0.1 mg. per 100 grams of rat twice a week for 13 weeks. Thus the total dose per rat ranged between 4.0 and 5.5 mg. Rats were allowed to live as long as possible and most of them were killed when they were between 450 and 600 days old.

Major Findings: Rats that died or were killed early in the experiment developed hyperplastic nodules or small hepatomas in the liver; those that lived more than 500 days showed multiple hepatomas or carcinoma of the liver. Of the liver tumors transplanted, one has grown, and has the histologic pattern of the original tumor, a well-differentiated hepatic cell carcinoma.

Significance: The hazard which aflatoxin may present to man is unknown. It is known that aflatoxin is the toxic principle of aspergillus flavus which in various parts of the world is a contaminant of peanuts and peanut meal eaten by people in Africa, for instance, where the incidence of liver cancer is high. Man might react like the rat, in which no toxic effects

of the aflatoxin was observed, but in which liver tumors appeared after a long interval. The role that consumption of fungal products may play in the etiology of a fatal disease of young adults is obviously worth investigation.

Proposed Course: Examination of histologic sections from this experiment will be completed and the results prepared for publication. Because of the possibility that aflatoxin may be hydrolyzed in alcoholic solution, another experiment is in progress in which dimethylformamide was used as the vehicle.

F. Transplantation of Tumors

Objectives: To carry interesting or unusual tumors in transplant for possible future study in this laboratory or by other investigators.

Methods and Major Findings: The following tumors are carried by subcutaneous, intraperitoneal, or intramuscular injection:

494..Adrenal cortical carcinoma, OM/N rat. Tumor has been used by several investigators for hormone research and is currently being studied by Dr. Max Sweat of the University of Utah, by Dr. Robert L. Ney of Vanderbilt University, and in tissue culture by Dr. L.M. Edelstein of the New England Medical Center, Boston.

555..Gastric adenocarcinoma induced by 2,7-FAA in ACI/N rat. Tumor develops metaplastic bone, bone marrow and cartilage in center.

328..Gastric adenocarcinoma, C57BL mouse, induced by intramural injection of 3-MCA into the wall of the stomach. Mice in which this tumor has grown for a long time develop hypervolemia. Currently being studied is tissue culture by Dr. L.M. Edelstein of New England Medical Center, Boston.

630..Malignant mesothelioma of the genital omentum of Buffalo rat whose mother received 2,7-FAA during pregnancy and lactation. No other similar tumor known to be transplantable.

639..Gastric tumor, possibly malignant neurilemoma, induced by the intragastric administration of Methylnitrosourea in BUF/N rat.

642..Glioma, induced in the brain of a BUF/N rat by the intragastric administration of Methylnitrosourea.

586..Skin appendage tumor induced in ACI/N rat by oral administration of 2,7-FAA, being studied by Dr. Shu Wen How and in tissue culture by Dr. L.M. Edelstein of the New England Medical Center, Boston.

599..Adenocarcinoma of the glandular stomach induced by oral administration of 2,7-FAA in ACI/N rat.

638..Malignant neurilemoma of the trigeminal nerve that originated in a Buffalo rat that had received methylnitrosourea intragastrically by stomach tube.

645..Carcinoma of the glandular stomach that originated in an untreated Mastomys. The first successful transplant of a tumor of this type.

654..Hepatic cell carcinoma that originated in a Buffalo rat that received aflatoxin in alcohol intragastrically by stomach tube.

657..Rhabdomyosarcoma. Developed spontaneously in the dorsal spinal muscles of a 4 month old WN rat. Tumor is now in its 3rd transplant generation and maintains its original pattern of malignant striated muscle cells.

Significance: The study of transplantable tumors continues to be a useful tool for investigators interested in hormone research, tissue culture, biochemistry and transformation of tumor type during successive transplant generations.

Proposed Course: To maintain the tumors in transplant and make them available to other investigators.

Publications

1. Snell, K.C., and Stewart, H.L.: Neoplastic and non-neoplastic renal diseases in Praomys (Mastomys) natalensis. J. Nat. Cancer Inst. 39: 95-117, 1967.

2. Stewart, H.L., and Snell, K.C.: Thymomas and thymic hyperplasia in Praomys (Mastomys) natalensis. Concomitant myositis, myocarditis, and sialodacryoadenitis. J. Nat. Cancer Inst. (in press)

Serial No. NCI 524
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Studies on interferon production and action

Previous Serial Number: Same

Principal Investigator: Robert M. Friedman

Other Investigators: Mary Ann Guggenheim, Alan S. Rabson

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 2
Professional: 1
Other: 1

Project Description:

Objective: To study the mechanism of action of interferon and its possible role in viral oncogenesis.

Methods: Standard virologic methods for virus and interferon assay. Standard biochemical assays for protein and RNA which involve the use of radioisotopes, radioautography, base ratio analysis, sucrose density gradient analysis, and electron microscopy.

Findings: The action of interferon is basically a two-step process. The first step involves attachment of interferon to cells and takes place at 1°C. The second requires RNA and protein synthesis and needs a period of incubation at 37°.

The first step has been investigated by incubating cells with interferon at 1°C, washing and observing the establishment of antiviral activity. Interferon rapidly and irreversibly attaches to cells. The reaction probably involves a chemical equilibrium between interferon bound to a surface site and free interferon since a maximum interferon response is reached in the system with a 10-20 min. incubation at 1°C. The site is probably on the cell surface as treatment with trypsin after interferon has been washed off completely inhibits the establishment of antiviral activity. In this respect interferon resembles other biologically active polypeptides such as TSH, ACTH, insulin, and some of the colicins.

The second step in interferon action would appear to involve an antiviral protein which inhibits the production of virus specific proteins. In the case of RNA viruses treatment with interferon has been found to block the formation of virus double stranded RNA by labelled parental input virus. This is of significance bexause the double stranded RNA would appear to be the template on which new viral RNA is formed. The passage of parental virus into the double stranded form requires production of a specific viral enzyme, an RNA polymerase. Therefore, the inability of the virus to pass into this form is a manifestation of interferon's ability to block viral protein synthesis.

Interferon fails, however, to block the passage of the virus into a ribonuclease sensitive 30S form very soon after infection. Since this form probably represents a conformational change in viral RNA configuration it is wholly consistent that interferon has no effect on its formation.

Studies on interferon production and action employing cell fusion techniques are also being carried out. These studies show that following fusion the repressed genetic information present in the avian red cell nucleus may be activated. Also following fusion cells appear to become insensitive to interferon's antiviral activity.

Significance: Interferon would appear to be exclusively an inhibitor of viral RNA production. It may be useful in control of oncogenic viruses. It may also be of some use in indicating differences between cell and viral RNA and in pinpointing sites of cellular RNA control mechanisms. In addition, interferon also is an interesting substance to employ in studying induced protein synthesis in animal cells.

Proposed Course: Attempts will be made to show direct effects of interferon on viral protein synthesis.

Publications:

1. Friedman, R.M.: Interferon binding: the first step in establishment of antiviral activity. Science 156: 1760, 1967.

2. Friedman, R.M., Fantes, K. H., Levy, H.B. and Carter, W.B.: Interferon action on parental Semliki Forest virus ribonucleic acid. J. Virol. 1: 1168, 1967.

3. Guggenheim, M.A., Friedman, R.M. and Rabson, A.S.: Interferon production in chick erythrocytes activated by cell fusion. Science 159: 542, 1968.

4. Baron, S., Bucker, C. E., Levy, H.B. and Friedman, R.M.: Some factors affecting the interferon induced antiviral state. Proc. Soc. Exp. Biol. Med. 125: 1320, 1967.

5. Friedman, R.M.: Interferon and virus infections. Am. J. Nursing. 1968.

Serial No. NCI 554
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Studies on replication of an RNA virus

Previous Serial Number: Same

Principal Investigator: Robert M. Friedman

Other Investigators: Philip M. Grimley, Robert Stern, Ira Pastan

Cooperating Units: NIDR; NIAMD

Man Years (July 1, 1967 - June 30, 1968)
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objective: To study the replicative process of an RNA virus.

Methods: Viral plaque assays. RNA extractions. Sucrose gradients. Scintillation counting. Electron microscopy. Base ratio analysis. Lipid analysis. Column chromatography.

Major Findings: Four replicative RNA forms of an arbovirus have been found and characterized. Their formation is blocked by interferon, but not by actinomycin D.

One form sediments in a sucrose gradient at 20S and is ribonuclease resistant. Another form sediments at 26S, is RNAse sensitive and appears to be related to the single stranded (42S) RNA of the virus. The true double stranded base paired viral RNA sediments at 16S. The last form to be found was a polydisperse form with characteristics of both single and double stranded RNA, probably a replicative intermediate.

The three forms of virua RNA in infected cells are associated with distinct cytoplasmic particles. A membraneous particle contains all three forms of RNA but a high percentage of the RNA appears to be in the replicative form and replicative intermediate form. A 65S particle contains only the 26S form. Its function is at present unknown. A 140S particle contains only the 42S form of viral RNA. This particle would appear to be an intracellular form of virus. Electromicrographs show that the 140S particle is a viral form apparently lacking an outer envelope.

Analysis of the four bases present in the RNA forms indicate that the 26S and 42S forms have identical base ratios. The 20S form has a base ratio consistent with its being a triplex composed of 2 strands of parental RNA together with one complementary strand and the replicative form is a base paired RNA structure. Knowledge of these multiple RNA forms has permitted us to investigate how they are related to virus protein synthesis. We have also been studying the number of proteins coded for by the genetic information of the virus. In brief, protein synthesis by the virus is carried out by nascent RNA which has not yet disengaged from its template. Analysis of virus coded proteins has so far turned up only three. This may be of special interest as it is then a very useful genetic unit.

Knowledge of the viral replication mechanism has also been of use in studying the action of inhibitors of virus growth. Studies on interferon have been previously discussed. In addition, studies with a purified bacterial enzyme, phospholipase C have shown that it markedly inhibits virus replication by damaging cell membranes. The latter action is not serious enough to disrupt cell biochemical processes.

Significance: These forms of RNA may be important to any understanding of the replication of RNA viruses. It is hoped that the findings in this study will be of use in later studies of RNA tumor viruses.

Proposed Course: Investigate the cellular location of virus protein synthesis and of the RNA forms.

Publications:

1. Friedman, R.M. and Berezesky, I.: Cytoplasmic fractions associated with Semliki Forest virus-RNA replication. J. Virol. 1: 374, 1967.

2. Friedman, R. M.: Protein synthesis directed by an arbovirus. J. Virol. 2: 26, 1968.

3. Friedman, R. M.: Replicative intermediary of an arbovirus. J. Virol. 2: In press.

4. Pastan, I. and Friedman, R. M.: Inhibition of cell phospholipid synthesis by actinomycin D. Science. In press.

5. Friedman, R.M. and Pastan, I.: Specific inhibition of virus growth by phospholipase C. Proc. Nat. Acad. Sc. In press.

Serial No. NCI 564
1. Laboratory of Pathology
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Interferon production by non viral substances

Previous Serial Number: Same

Principal Investigator: Robert M. Friedman

Other Investigators: Herbert L. Cooper and Yale Topper

Cooperating Units: HGB:NIDR; LBM:NIAMD

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/4
Other: 1/4

Project Description:

Objective: To study interferon production induced by non-viral materials especially by human mitogens.

Methods: Cultures of human and mouse cells. Interferon assay and characterization. RNA extractions.

Results: Phytohemagglutinin, poke weed mitogen, and streptolysin-O cell mitogens in human lymphocyte cell cultures also induce interferon production in these cultures. Interferon is produced very rapidly in response to addition of mitogen and production turns off promptly upon its removal.

Interferon may also be produced in other system where DNA synthesis is stimulated.

Significance: These studies indicate that interferon production may be a concomitant of cell growth. They may also be a useful way to study induced processes in cells.

Publications:

1. Friedman, R.M. and Cooper, H. L.: Interferon production in human lymphocyte cultures following treatment with mitogens. Proc. Soc. Exp. Biol. Med. 125: 901, 1967.

Serial No. NCI 503
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Collaborative research requiring pathologic anatomy

Previous Serial Number: Same

Principal Investigator: Thelma B. Dunn

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Objectives: To collaborate with and advise other investigators at the National Cancer Institute and elsewhere in phases of research problems requiring experience in animal pathology.

Methods: This may consist of (1) full responsibility and separate publication as senior author of material reviewed in connection with work of another investigator; (2) review and description of animals coming to autopsy, and publication of the findings as a separate section of a paper describing the entire experiment; (3) review and diagnosis of tumors found by investigator in course of experiments, the information to be used in a tabulation of results; (4) occasional consultation and advice on pathologic aspects or material in an experiment; (5) diagnosis of microscopic slides sent from other institutions.

Major Findings: (1) Work done with Dr. Virginia Evans on transfers of tissue culture cells to the anterior chamber of the eye is continuing. This will be reported in detail by Dr. Evans. Considerable variation is observed depending upon material injected. A similar study has been made with Dr. Katherine Sandford using anterior chamber transplants of rat's eyes. Tumors found in experiments of Dr. Andervont, Dr. Deringer, and others have been diagnosed. (2) A project of particular interest on amyloidosis is now in progress with Dr. Andervont.

Significance: Pathologic review is indispensable to many types of cancer research. This is best carried out by a specialist in pathologic anatomy of particular animal species, since with the present complexities of research a single investigator cannot know this field and his own specialty very well.

Proposed Course: This work will continue as in the past. When, as a result of many observations, enough information is accumulated regarding any subject, publications will be made.

The study on amyloidosis with Dr. Andervont is based on the development of an inbred line of wild mice with a high incidence of amyloidosis. The wild mice have been crossed with the high amyloid strain A, and low amyloid BALB/c mice in the hope of disclosing genetic factors. The hybrid mice are being kept until they reach a significant age.

Serial No. NCI 504
1. Laboratory of Pathology
2. Cancer Ind. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: An electron microscopic investigation of normal and pathologic tissue

Previous Serial Number: Same

Principal Investigator: William G. Banfield

Other Investigators: George Kasnic, John H. Blackwell

Cooperating Units: Microbiological Associates

Man Years (July 1, 1967 - June 30, 1968)
Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives:

1. To describe and interpret the ultrastructure of selected pathologic states and to compare the results with suitable controls.

2. To look for and study the inciting agent or accompanying agent in neoplastic and other disease states using human, animal and tissue culture material.

Major Findings:

1. An abstract of the work on epizootic diarrhea of infant mice (EDIM) is attached. We have extended this through enzyme studies which indicate that EDIM is probably an RNA virus. The manuscript is almost ready for submission to Virology. In addition to tubules, we have recently found a fibril associated with this virus.

The small dense granule described by us in the elementary body of molluscum contagiosum is present in the Shope fibroma elementary body which we are now using to find out the granules' composition and possible function.

3. Dr. Stanton and technician have been trained to use our microscope. His work will be reported by him.

Proposed Course:

1. EDIM virus will not grow in tissue culture. We may try organ culture to study its life cycle further.

2. Electron microscopic histochemical methods are being applied to lesions of Shope fibroma with special attention directed toward the dense granule in the elementary body.

3. Dr. Dawe will be trained to use the electron microscope. I have been doing some work with him which will be described in his report.

Significance:

1. The EDIM virus with its many developmental forms is an ideal subject in which to work out the complete ultrastructural histology of the virus-life cycle. This should explain the relationship to viral development of associated structures such as tubules and fibrils seen in other viruses. Morphologically EDIM does not fall neatly into any of the RNA groups.

2. The dense granule is an elementary body organelle, present in some of the pox viruses. It is important to determine its composition and function and to what extent it may be present in the structure of other viruses.

Publications:

Norris, G. F., Banfield, W. G. and Chalifoux, H.D.: Tissue processor for automatic dehydration and infiltration of small specimens. Science Tools 14: 13, 1967.

Serial No. NCI 504

TWENTY-FIFTH ANNUAL EMSA MEETING
August 29-September 1, 1967
Chicago, Ill.

FURTHER OBSERVATIONS ON THE VIRUS OF EPIZOOTIC DIARRHEA OF INFANT MICE. AN ELECTRON MICROSCOPIC STUDY

W. G. Banfield, G. Kasnic, Jr. and J. H. Blackwell

Laboratory of Pathology, National Cancer Institute, Bethesda, Maryland 20014 and Microbiological Associates, Inc., Washington, D.C. 20014

An ultrastructural study of the intestinal epithelium of mice infected with the agent of epizootic diarrhea of infant mice (EDIM virus) was first performed by Adams and Kraft (1). We have extended their observations and have found developmental forms of the virus and associated structures not reported by them.

Three-day-old NLM strain mice were infected with EDIM virus and killed 48 to 168 hours later. Specimens of bowel were fixed in glutaraldehyde, post fixed in osmium tetroxide and embedded in epon. Sections were stained with uranyl magnesium acetate followed by lead citrate and examined in an updated RCA EMU-3F electron microscope.

The cells containing virus particles (infected) are at the tips of the villi and occur throughout the intestine from duodenum through colon. All developmental forms of the virus are present from 48 to 168 hours after infection. Figure 1 is of cells without virus particles and figure 2 is of an infected cell. The nucleus and cytoplasm of the infected cells appear clearer than the cells without virus particles. This seemed to be the result of a loss of granularity of the interchromatic material of the nucleus and a loss of free and bound ribosomes in the cytoplasm. There also may be an increase in nuclear sap and possibly a cytoplasmic edema. The most fully developed virus particle is most often ovoid, as small as 705 A in one diameter and as large as 1128 A in the other with a ratio of major to minor axis equaling 1 to 1.3, usually about 1.2. The particle has an outer coat~185 A in width and a central body~560 A in diameter which may or may not contain a dense nucleoid~270 A in diameter. The particle begins development within islands of viroplasm, Fig. 2 and 3, which are masses of dense, finely granular, rather fuzzy cords within the cytoplasm. Within the cords are somewhat elliptical central bodies with diameters from 450-560 A. Within the central bodies a dense nucleoid may develop 240-280 A in diameter. It is centrally placed and does not fill the central body. Aggregates of central bodies containing nucleoids are not uncommon (Fig. 4). The viroplasm seems to border endoplasmic reticulum which has lost most of its RNP granules adjacent to the viroplasm. Central bodies pass through the membrane of the endoplasmic reticulum and in doing so acquire another coat (Fig. 4). However, central bodies without the coat are present in the cytoplasm and endoplasmic reticulum.

Within the nucleus and cytoplasm of infected cells tubules are often present in varying number and length. Within the nucleus they contain a single membrane and are 540-620 A in width. When they extend through the nuclear envelope, that portion penetrating the envelope acquires an additional membrane. In the cytoplasm, single and double membraned tubules are seen. The overall diameter of the double membraned tubule is from 840-940 A. A double membrane may be acquired when a single membraned tubule passes into the cisterna of the endoplasmic reticulum from the cytoplasm. Tubules have also been seen within the viroplasm. Though Adams and Kraft found viroplasm and tubules rare, we did not. In our material tubules were common and seemed to be associated in some way with the normal life cycle of the virus.

1. Adams, W. R. and L. M. Kraft: Am. J. Path. In press 1967

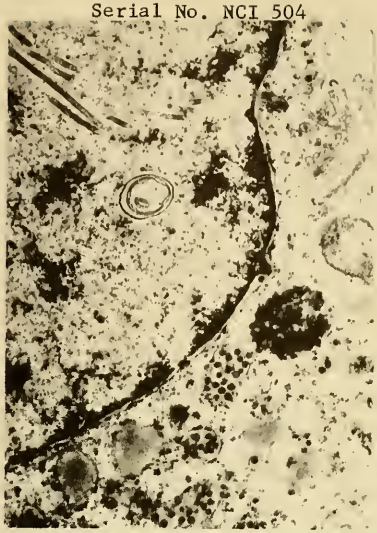
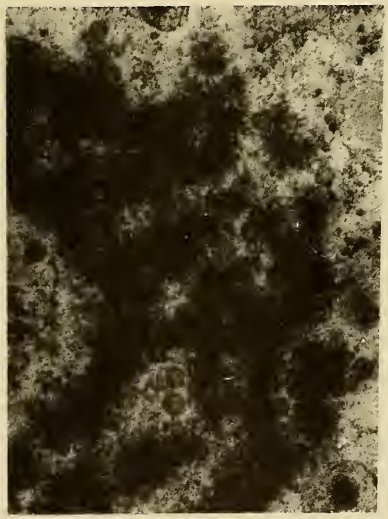


Figure 1. Cell free of virus particles. Note abundance of cytoplasmic ribosomes and granularity of nucleus as compared to Figure 2. 21,000X.

Figure 2. Portion of cell with cytoplasm containing virus particles in cisternae of the endoplasmic reticulum. The dense cytoplasmic mass is viroplasm. Nucleus contains tubules and a membranous inclusion. 16,800X.



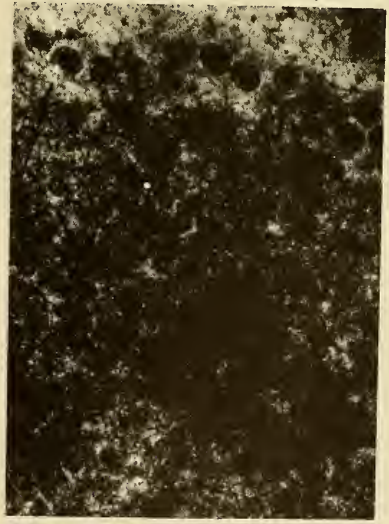


Figure 3. Viroplasm containing central bodies (arrow). 71,200X

Figure 4. Viroplasm containing mass of central bodies with nucleoids. A central body at the arrow is only partially covered by a coat. Others outside the viroplasm have a complete coat and probably lie within the cisterna of the endoplasmic reticulum. 71,300X.

Serial No. NCI 504a
1. Laboratory of Pathology
2. Cancer Ind. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Contagious tumors

Previous Serial Number: Same

Principal Investigator: Same

Principal Investigator: William G. Banfield

Other Investigators: M. Ohmori, G. Kasnic, C. K. Lee, Melpar, Inc.

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives: To study contagious tumors and attempt to find the mode and outline the parameters of contagion.

Major Findings: 1. The transmission of the hamster reticulum cell sarcoma TM by Aëdes aegypti was confirmed. There was no transmission of TM by culex pipiens quinque-fasciatus, Aëdes triseriatus or culex tarsalis. Two other hamster reticulum cell sarcomas, HaTU and TQ, failed to be transmitted by Aëdes aegypti.

2. Dr. Ohmori was unable to confirm the transmission of Shope fibroma virus by Aëdes aegypti.

Significance: Tumor transfer by transfer of cells may operate in man especially in tumors with leukemic manifestations or with cells in the blood stream.

Proposed Course: 1. In discussions with Dr. G. Nicholas Rogentine, Dr. Robert Miller and Dr. Joseph Fraumeni, we will try to work out an epidemiologic approach to the problem of cell transmission of tumors in man.

2. Dr. Paul Woke is studying the mechanism of mammalian cell transmission by the mosquito through an NIH grant.

3. As an outgrowth of the attempt to study development of Shope fibroma virus in the mosquito with the electron microscope, Dr. Ohmori will compile an atlas of the normal adult Aëdes aegypti from his micrographs of normal control mosquitoes on his return to Japan.

Serial No. NCI 504b
1. Laboratory of Pathology
2. Cancer Ind. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: An investigation of the collagenous connective tissues and elastin

Previous Serial Number: Same

Principal Investigator: William G. Banfield

Other Investigators: C.K. Lee, E. J. Miller, G.H. Stemmermann, S.W. Nielsen

Cooperating Units: NIDR; Kuakini Hospital & Home, Honolulu, Hawaii; Dept. of Animal Dis., Univ. of Connecticut, Storrs,Conn.

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: To elucidate the normal and abnormal structure of elastin and collagen.

Major Findings: An electron microscopic study was made of a benign connective tissue tumor, elastofibroma, characterized in light microscopy by "fibers, globes, and irregular masses" which take the elastic tissue stains. These masses were found by electron microscopy to consist of degenerating fibrils, often surrounding an elastic fiber core which was removed by elastase digestion. A matrix material surrounding the degenerating fibrils was also removed by elastase digestion. This material may have represented an elastic staining component of the fibrillar masses and could account for the disappearance of elastic staining from elastase-digested paraffin sections in light microscopy. These findings will be published in the May issue of JNCI.

Significance: 1. Quality information can be obtained from formalin fixed tissue even after long storage in formalin.

2. This is one of two unique instances of collagen pathology at the electron microscope level

Proposed Course: 1. Follow age changes with the electron microscope in elastic fibers of human skin.

2. Follow the phylogenetic development of elastin by light and electron microscopy.

3. Study the degeneration of collagen in mastocytomas by electron microscopy, Hottendorf, G. H. and Nielsen, S. W.: Collagen necrosis in canine mastocytoma. Am. J. Path. 49, 501-513, 1966.

4. Attempt to differentiate elastin from collagen in the electron probe with the view of studying elastin by elemental analysis.

Publications:

1. Banfield, W. G. and Lee, C. K.: Elastofibroma: an electron microscopic study. J. Nat. Cancer Inst. 40: 1968. In press.

Serial No. NCI 505
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Pulmonary carcinogenesis: animal studies related to cancer in man

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton

Other Investigators: R. Blackwell, E. Miller, C. Wrench, J. Albrecht & staff

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives: Experiments are continuing to determine factors that modify the responses of pulmonary tissue to chemical carcinogens and to determine the carcinogenicity of materials in the lungs of rodents that may be carcinogenic for man.

Major Findings: Work during fiscal year 1968 has centered on the study of various types of asbestos implanted in the lung or on the pleural surfaces of rats.

Pulmonary implants of beeswax pellets containing 10 mgms of either Cope blue crocidolite, amosite, Canadian chrysotile, or Rhodesian chrysotile have shown no indication of carcinogenicity after residing in the lung for 24 months. In contrast, similar beeswax pellets containing 50 micrograms of 3-MCA show evidence of prosoplasia and occasionally the development of carcinosarcomas. At higher doses 3-MCA shows a gradation of response from simple keratinized cysts through epidermoid cysts with local invasion to undifferentiated epidermoid carcinomas, the latter developing in over 70% of rats in less than 12 months at a dose of 2 mgms/rat or higher. The observations with 3-MCA indicate that this method should be a valuable method for detecting potential carcinogens in the lung.As noted in previous reports, a number of substances are in process of being tested.

Further work with crocidolite asbestos incorporated in gelatin-saturated fibrous glass pads placed on the pleural surface has indicated that the fibrous glass tends to form a stabile scaffold for growth of granulation tissue from the pleura and may act as an in vivo tissue culture milieu that encourages the neoplastic conversion of cells. Within 23 months 75% of the rats treated with 40 mgms of asbestos have developed mesothelial sarcomas roughly distributed betwen biphasic, osteogenic, and polymorphous types. Of particular interest is the fact that controls bearing the fibrous glass pads without asbestos have not developed tumors indicating in part that asbestos either because of its particular physical or chemical properties is actively carcinogenic.

The development of a transplantable epidermoid carcinoma of the lung in Fisher rats gave us the opportunity of testing a hypothesis that we considered possible, namely that critical subcellular components of neoplastic cell might be transferable to normal cells if given the right opportunity. In collaboration with Dr. A. Mulay, DNA and RNA were extracted from the tumor cells, suspended in high concentration in the fluorocarbon, herachloro-tetrachlorobutane, and injected intravenously. Theoretically, the regenerating pulmonary epithelial cells at the sites of pulmonary infarction that are induced by the latter compound, were in the optimum state to utilize these nucleic acid fractions from neoplastic cells of a common origin and the nucleic acid fractions were released slowly into the adjacent pulmonary tissues at the site of pulmonary infarction. Since the nucleic acids were derived from neoplastic pulmonary epithelial cells of the same strain, and since the pulmonary epithelial cells at these sites were in an optimum state of injury and regeneration to utilize these nucleic acids, two special conditions were met for inducing tumors by nucleic acid extracts. As yet, none of the rats has developed lung tumors, but only a few tests have been made with relatively unsatisfactory nucleic acid preparations.

Proposed Course: The value of determining the critical level of a given carcinogen in a standard pellet lies in its use in determining potential carcinogenicity of other compounds. For example, can particulates such as nickel, iron oxide or tobacco enhance the carcinogenicity of a subcarcinogenic dose of MCA? In the relatively insoluble beeswax pellet system, the role that these compounds might play in localization by surface adsorption is essentially eliminated so that other factors related to enhancement can be determined. Efforts to detect carcinogenic activity of a variety of materials (arsenic, tobacco and other cigarette components, asbestos, nickel, titanium, air pollutants) are continuing. Experimentation with carcinogenesis in the pleural cavity will occupy the major part of our time. We intend to test various forms of fibrous glass used in homes and industry for carcinogenicity. The newer fibrous glasses, and those used in our original experiments are coated with a phenolic resin to enhance plasticity. Old fibrous glasses used in house insulation, Christmas tree decorations, etc., are not. Wagner has previously shown that pulverized silica is carcinogenic. It would be interesting to learn whether silica or asbestos coated with the fibrous glass resin would protect against carcinogenicity. More than likely this would solve the problem for the asbestos industry if it were economically feasible.

Publications:

1. Stanton, M.F. and Blackwell, R.: Experimental pulmonary carcinogenesis with asbestos. J. Am. Ind. Hyg. Assoc. 1968. (Abstract March-April 1968)

Serial No. NCI 510
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: A guide to the normal and pathologic anatomy of the laboratory mouse.

Previous Serial Number: Same

Principal Investigator: Thelma B. Dunn

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Objectives: This is a continuation of work begun a number years ago. Because of the magnitude of the project it now seems advisable to undertake it piecemeal. A review of endocrine organs in the mouse is now in progress and material and references on the adrenal gland have been accumulated. Some of this has been made available to Dr. Victor Swaen, a guest worker from Amsterdam, who is preparing a paper on the adrenal cortex in BALB/c mice.

Methods: Personal observations on material submitted from different sources, planned experimental procedures in inbred mice, histologic study of untreated mice at various ages, systematic review of the literature.

Major Findings: Two talks were given at a Conference on Spontaneous Diseases of Rats and Mice held in April 1966 under the auspices of the Nuffield Foundation in England. Material for these talks was obtained from data already accumulated for the proposed guide. Papers were published in a Report on the Conference, edited by Cotchin, and published recently.

Significance: Since the mouse is so widely used in many areas of research, an understanding of the normal and pathologic anatomy is imperative, if intelligent conclusions are to be reached regarding the effect of various agents. Also since hormonal studies are an important part of cancer research it is desirable that reliable information be available regarding the endocrine and reproductive organs of inbred strains of mice.

Proposed Course: Progress has been slow because of many interruptions. It is hoped that a final draft on the adrenal gland can be completed during the coming year.

Serial No. NCI 514
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Induced and spontaneous neoplasms in laboratory animals. Factors that influence their incidence and the chemical changes induced by the neoplasms.

Previous Serial Number: Same

Principal Investigator: Ambadas S. Mulay

Other Investigators: R. W. O'Gara and V. S. Waravdekar (Not NIH)

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1
Professional: 1/2
Other: 1/2

Project Description:

A. Objective: To study the effect of riboflavin analog, 6,7-dimethyl-9-(2'-aceloxyethly)-isoalloxazine (U-2112) on azo dye hepatocarcinogenesis.

Methods: Male rats were fed powdered Purina laboratory chow containing U-2112, hepatocarcinogen, N,N-dimethyl-p-phenylazoaniline (DAB) or both, in various concentrations. A few animals from each group are killed at regular intervals and tissues studied for chemical and histological changes.

Major Findings: Results show that all concentrations of U-2112 tried--100, 50 and 10 mg % were too toxic in conjunction with 0.18% DAB. Most of the animals on this combination died within a month. U-2112 alone, 100 mg % does not induce any significant histological changes in the liver. A combination of 50 mg % U-2112 and 0.12% DAB induces many liver changes among which cholangeofibrosis is most prominent in the rats on this diet for 4 months or longer. 100 mg % U-2112 for first two weeks and 50 mg % for the rest of 4 months in conjunction with 0.12% DAB induced hepatomas in 40% of the rats and cholangeofibrosis in the rest. A paper reporting these findings has been submitted to J.N.C.I. for publication.

Significance: Riboflavin analog U-2112 has been reported to regress established tumors, and reduce tumor incidence in primary carcinogenesis. Our

results show that U-2112 increases the incidence of tumors in DAB carcinogenesis. Most probable mechanism--occupation by U-2112, riboflavin sites in enzymes necessary for detoxification of the carcinogen, a riboflavin antagonist.

Proposed Course: No further work is planned on this project.

Publications: 1. Mulay, A. S. and O'Gara, R. W. "Enhancing effect of a riboflavin analog on azo-dye carcinogenesis in rats". (In Press) J.N.C.I. Vol. 40: April 1968.

B. Objective: To study the effect of fatty acids and fats, irradiated with ultra-violet light, on the chemistry and histology of the liver of rats, when given alone or in conjunction with a hepatocarcinogen.

Methods: Rats when fed powdered Purina laboratory chow containing a subcarcinogenic dose of N,N-dimethyl-p-phenylazoaniline (DAB) for 4 months and the animals were killed at the end of 4th, 5th and 6th month. Linolenic acid was irradiated with U-V light, then extracted with water, and water extract concentrated to contain known number of thiobarbituric acid units (TBA units) per ml. Rats received none, one or more I.P. injections of the extract 1 ml/injection at monthly intervals. Some rats had DAB only. Some had extract only and some had both. Groups differed both in number of injections received (1 to 4) and in TBA units/Kg/inj. Biochemical, histochemical and histological studies were made on the livers and all other tissues were examined for possible lesions.

Major Findings: Injection of U-V-extracts with high TBA units/Kg were very toxic and killed the rats in 2 to 3 days, medium concentration of TBA units/Kg prolonged the life of rats to about 3 months and the animals died of internal hemorrhages, adhesions, and intestinal obstructions. Rats receiving low concentration of TBA units in conjunction with subcarcinogenic dose of DBA in the diet induced extensive cholangeofibrosis in rats receiving 3 or 4 injections and killed at the end of 5th or 6th month. Some of the livers had small hepatoma nodules. Enzyme systems associated with organelles of the cytoplasmic origin showed only 60-7 % activity, in livers which developed cholangeofibrosis and hepatoma nodules, of the control livers.

Significance: Ultra violet irradiation of fatty acid (fat) produces substances which are toxic to rats. Toxicity of UV-extract to hepatic cells, it is concluded, impairs their capacity to detoxify even subcarcinogenic dose of DAB, thus increasing the possibility of carcinogenesis, where none existed before.

Proposed Course: No further work is planned on this project. A paper based on these findings is being prepared for publication.

C. Objective: To establish a dose response curve for carcinogen N,N-dimethyl-p-(m-tolylazo)-aniline (mé-DAB) when fed in conjunction with complete diet of powdered Purina laboratory chow.

Methods: Accurately weighed carcinogen was incorporated either in semisynthetic or in powdered Purina laboratory chow and fed to the animals for 4 months and experiment terminated 2 months later. Male rats of Osborne-Mendel, NIH Black, and Marshell-520 strain and females of Osborne-Mendel strains were used in these experiments. Animals were examined every day and some were killed at regular intervals for biochemical and histological studies.

Major Findings: It takes higher concentration of mé-DAB to induce hepatomas in rats on Purina laboratory chow diet than those on semisynthetic diet. 0.06% mé-DAB gives high incidence of hepatomas in rats on semisynthetic diet. On Purina laboratory chow diet, it takes 0.09 % mé-DAB for male and 0.12 % mé-DAB for female Osborne-Mendel rats to get similar incidence of hepatomas. 0.06 % mé-DAB gives about 20 % cholangeofibrosis in livers of male and no significant change in those of female rats. 0.09 % mé-DAB gives some cholangeofibrosis and one (10 %) hyperplastic nodule in female rats. Incidence and histology was similar in all three strains of rats--Osborne-Mendel, NIH Black, and Marshell-520. Three of the hepatomas induced in NIH-black rats were successfully propagated through 8 transplant generations.

Significance: Dose response curves for me-DAB fed in Purina laboratory chow to male and female Osborne-Mendel rats have been established. These curves could be used as base lines for the study of the effects of other agents including carcinogens on mé-DAB carcinogenesis.

Proposed Course: No further experimental work is planned on this project at this time. Work of tabulating and evaluating the results is in progress. A paper based on these findings will be prepared.

D. Objective: To study the effect of N,Nitrosodiethylamine (DENA) a carcinogen, on N,N-dimethyl-p-phenylazoaniline (DAB) hepatocarcinogenesis in rat.

Methods: Rats received 5 mg % DENA in drinking water for 4 months. Some of these animals received a subcarcinogenic dose of DAB (0.12 %) in Purina laboratory chow diet for the same period. Another group of rats received only 0.12% DAB in their chow diet. The experiments were terminated 2 months later and the animals killed for biochemical and histological studies.

Major Findings: Preliminary results show that DENA alone induces cholangeofibrosis and hemorrhagic lesions of the liver in high percentage of rats that survive the experimental period. The animals receiving combination treatment of DENA and DAB seem to show more hepatoma nodules and DAB alone shows very little change in liver histology.

Significance: Interaction of one carcinogen on the carcinogenic action of another may give some clues to their mode of action.

Proposed Course: Closer evaluation of the results, repeating the experiments where results are in doubt and then reporting the findings.

E. Objective: Study of dietary and environmental factors in azo dye carcinogenesis.

Methods: Rats were fed semisynthetic diet containing 0.06 % azo dye carcinogen N,N-dimethyl-p-phenylazoaniline (DAB). Different groups of animals differed from each other in their choline, pantothenic acid, niacine or fat content. Some animals had softwood bedding and decreased on hardwood bedding (Science 157: 1057; 1967)

Major Findings: Experiments are still in progress. No results to report at present.

Significance: These experiments may lead to some factors which influence the incidence of tumors under defined circumstances.

Proposed Course: Work will be continued in next fiscal year.

Serial No. NCI 516b
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Induction and histopathological analysis of bone tumors in rats: animal studies related to cancer in man

Previous Serial Number: Same

Principal Investigatos: Mearl F. Stanton

Other Investigators: R. Blackwell, E. Miller, J. Albrecht and staff

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives: Studies on fine structure and behavior of the transplantable tumors.

Major Findings: The diverse character of the tumors of bone induced with cupric-chelated H-OH-acetylaminofluorene and the mixture of several types of cells within a single tumor has motivated the establishment of 15 transplantable tumors from this source in order that some understanding of the origin and character of the tumors may be attained. Current studies suggest that progeny of an apparently uniform tumor may under some circumstances synthesize bony matrix while under other circumstances the tumor cell may synthesize cartilagenous matrix or form vascular spaces. Whether or not the tumors may also convert to blood forming elements is currently under study.

The tumors have been stored in liquid nitrogen and reestablished tumors of this type have been supplied to 3 outside investigators and 2 investigators at NIH. The similarity between the variety of tumors induced by this method and those induced with MSV (see NCI 516c) has motivated a rescreening of the tumors with the electron microscope for virus particles. Thus far none has been found but further work is needed before any conclusion can be made.

Proposed Course: Two courses are being followed. First, efforts are being directed to determine factors that might influence differentiation an and to learn whether the less differentiated tumors of osseous tissues can differentiate in more than one direction. Classical characteristics of differentiation are easy to identify in the tumors. The advantage of using tumors with known potential for differentiation and in early transplant generations should make these tumors particularly valuable for this type of study. The similarity between the tumors induced and those that occur in man offers a unique opportunity to determine by analogy, origin and behavioral patterns of bone tumors of man such as osteoclastomas, Ewing's tumor and the relationship between aneurysmal bone cysts and telangiectatic osteosarcomas.

Detailed descriptions of the fine structure of transplantable tumors are scarce and the present study may yield characteristics of value in differentiating tumors of mesenchymal origin.

Secondly, the fine structure of neoplastic cells of bony tissues are being studied. This program at the moment is handicapped by lack of an available electron microscope but prepared grids are available when the opportunity develops. It is perhaps of immediate interest that we learn whether these tumors possess virus, since osteogenic sarcomas can be induced in rats with both RNA and DNA viruses and there is an apparent increase in naturally occurring osteogenic sarcomas of the rat in other experiments in this laboratory which might suggest horizontal transmission of a virus.

Serial No. NCI 516c
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Viral Carcinogenesis, Problems of Immunity, Resistance, and Pathogenesis

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton

Other Investigators: Eliza Miller, Joseph Albrecht and staff

Cooperating Units: Dr. Lloyd Law and staff (NCI - 3200)

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives: Studies of histopathological and fine structure alterations related to the pathogenesis of disease induced by a murine sarcoma virus.

Major Findings: The histopathological changes in a select group of 150 mice have been studied and additionally 25 mice were serially sacrificed to detect early changes. To date we have concluded that the response to this virus is unlike that of any other virus. The early progressive changes are predominantly of a granulomatous character, but the lesions consistently contain a scattering of cells not typical of inflammation.

The study of lesions induced by murine sarcoma virus (Moloney strain) in both rats and mice has yielded several important findings which perhaps represent the "highest light" of this report.

The disease induced by this virus is initially an inflammatory granuloma in which atypical cells occur. This response occurs in many tissues although some tissue-selectivity seems to occur. Ultimately a small percentage of the lesions evolve into simple neoplasms generally of mesenchymal origin such as fibrosarcomas, hemangiosarcomas, osteogenic sarcomas and myogenic sarcomas, but one tumor, the MSB-1 tumor, clearly seems to be derived from mammary tissue of a strain BN rat. The unique reactive response to either the atypical cells or the murine sarcoma virus and its relation to leukemogenesis and such neoplasms in man (for example Kaposi's disease, Hodgkin's disease and the reticuloendotheliosis) that show similar reactive components make

this disease a particularly critical one in studies of viral carcinogenesis. Further studies in viral oncogenesis have centered on characterization of cell structure and cell alterations induced by infection with so-called "murine sarcoma virus" by electron microscopy. The general conclusion that can be made at this point is that certain unique alterations in cell organelle occur regardless of the type of cell that responds, and further that these characteristics are also common to those induced by the avian leukemia-sarcoma complex. Diagrammatic representation of these changes are included in this report as Figure 1.

Proposed Course: Further studies are being conducted to determine whether more epithelial tumors can be induced by this virus. Since it has been shown that the addition of a leukemogenic murine virus to a previously virus-free MSV tumor results in the recovery of a murine sarcoma virus, experiments will be designed for study with the electron microscope to detect possible mechanisms of this phenomenon.

Publications:

Papers entitled: Some Biologic, Immunogenic and Morphologic Effects in Mice Following Infection with a Murine Sarcoma Virus.

I. Biologic and Immunogenic Studies
II. Morphologic Studies

with Low, L.W. and Ting, R.C. will be published in the May issue of JNCI.

Paper entitled: The Fine Structure of Transplantable Tumor MSB-1: A Presumed Mammary Carcinoma Induced in the Strain BU Rat by Murine Sarcoma Virus is in preparation.

As participant: Criteria for the Determination of the "Normal" and "Leukotic" State in Cattle.--a statement prepared by the International Committee--JNCI, April 1968.

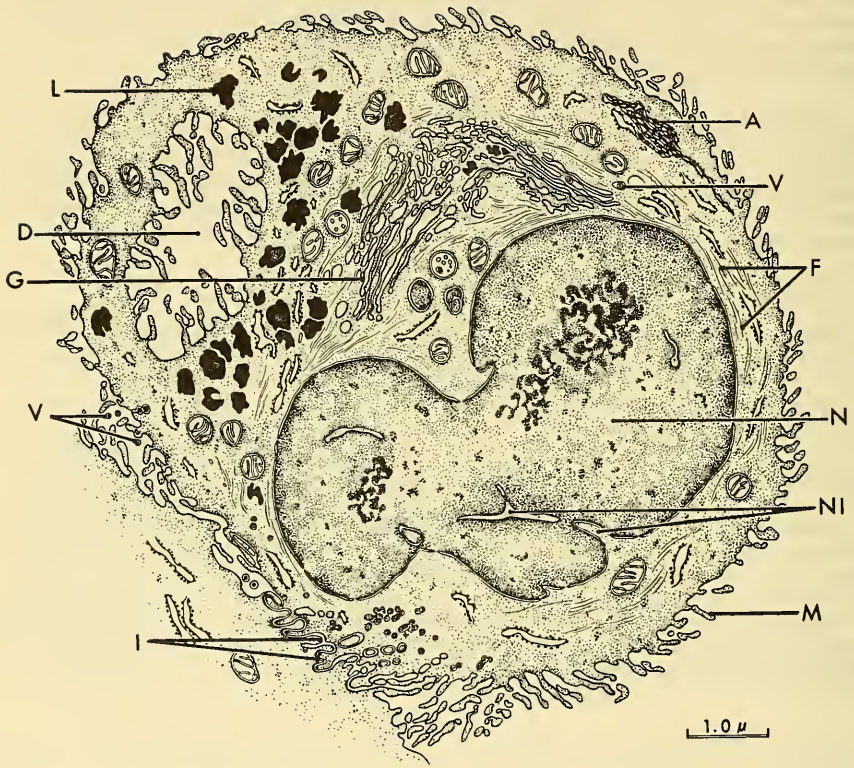


Fig. 1.--Diagrammatic Representation of MSB-1 Cell
A-Anullate lamellae; L-Lipid; V-Virus; F-Microfibrils; N-Nucleus; NI-Nuclear invaginations; M-Microvilli; I-Interdigitated microvilli; G-Paranuclear "Cell center"; D-Intracytoplasmic ductule.

Serial No. NCI 519
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Induction of neoplasms, selection of transplantable strains of tumors, and study of tumor and host chemistry

Previous Serial Number: Same

Principal Investigator: Ambadas S. Mulay

Other Investigators: William G. Banfield, Mearl F. Stanton

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)

Total: 1
Professional: 1/2
Other: 1/2

Project Description:

A. Objective: To induce lung tumors in rats, that could be transplanted.

Methods: (a) Osborne-Mendel rats were given subcutaneous injections of freshly prepared suspension of 4-nitroquinoline-N-oxide (NQO) in 10 % lecithin, twice a week. (b) Another group of Osborne-Mendel rats were tube fed a NQO preparation in lecithin, Triton 100 and DMSO, three times a week. (c) Marshell-520, Fisher and Wistar rats were also put on subcutaneous injections of NQO. (NQO has been reported to have induced lung tumors in rats and mice--Gann 57: 559, 1966). Animals are examined every week day for possible effects of the treatment on the general health of the rats and evidence of any tumor induction.

Major Findings: Experiments are still in progress. Osborne-Mendel rats with subcutaneous injections of NQO that came to autopsy showed no lung lesions but big subcutaneous tumors at the site of injection. Histologically they were fibrosarcomas and may be rhabdomyosarcomas.

Significance: Transplantable lung tumors could be very useful as a tool in the study of carcinomas.

Proposed Course: These studies will be continued in the next fiscal year.

B. Objective: To induce kidney tumors in rats developing a transplantable kidney tumor.

Methods: Rats, Osborne-Mendel, Fisher, and Marshell-520 are fed carcinogen N-Nitrosodimethylamine in Purina laboratory chow for various times and animals observed for signs of tumor induction and transplanting the induced tumors to establish transplant lines.

Major Findings: The experiments are still in progress. One induced kidney tumor in Osborne-Mendel rat was transplanted to 3 rats of the same strain. It is too early to determine the success of the transplant.

Significance: A transplantable kidney tumor could be a useful tool for the study of these tumors.

Proposed Course: This work will be continued next year.

C. Objective: Attempt to propagate a transplantable (non-viral) tumor with cell free nucleic acid preparations from the tumor.

Methods: Grow and harvest transplantable tumors, disintegrate tumor cells, extract nucleoproteins. Isolate desoxyribose nucleic acid and ribose nucleic acid. Treat them in various ways, and introduce them in the host by various routes, and examine the animals at regular intervals for tumor growth. Tumor nucleic acids thus prepared are also characterized by their physical and chemical properties like absorption coefficient, sedimentation characteristics, base composition, etc.

Major Findings: Five attempts have been made to propagate hamster tumor by inoculating hamsters with tumor nucleoproteins, by various routes. None of the attempts was successful. One attempt has been made to propagate rat lung tumor with tumor nucleoproteins. No sign of success yet.

Significance: The experiments, when successful, will provide good evidence of entry of mamalian nucleic acid into intact mammalian cells.

Proposed Course: There are no plans for more work on this project.

Serial No. NCI 521a
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Investigations on the mechanisms of tumor induction by polyoma virus in mice

Previous Serial Number: Same

Principal Investigator: Clyde J. Dawe

Other Investigators: Jacqueline Whang-Peng, William G. Banfield, Willie D. Morgan, Ernest Hearon

Cooperating Units: Medicine Branch, NCI

Man Years (July 1, 1967 - June 30, 1968)
Total: 1
Professional: 1
Other: 0

Project Description:

In this continuing investigation, work during the past year has been aimed chiefly toward learning more about the development-supporting effect (DSE), discovered by us last year in polyoma virus-induced salivary gland tumors. This effect is manifested by the morphogenesis and cytodifferentiation of rudimentary salivary gland epithelium when placed within growing PV-induced salivary gland tumor tissue. Thus the salivary gland tumor tissue appears to be able to function much the same as normal salivary mesenchyme in supporting development by the epithelium.

Our studies are of 5 sorts:

1. Extension of the observations of support of development by the tumor tissue, to determine the degree of specificity with regard to both the tumor and the rudimentary epithelium that responds.

2. An experiment to establish beyond any doubt the cell type of origin of PV-induced salivary tumors (epithelium vs. mesenchyme).

3. An electron microscopic study of development of normal salivary epithelium within PV-induced tumors. Development under these abnormal conditions is compared with that when normal epithelio-mesenchymal relationships are undisturbed.

4. Time-lapse phase contrast studies of the interaction between PV tumor and normal epithelium, as compared with the interaction between normal mesenchyme and normal epithelium.

5. Trans-filter studies of the response of normal salivary epithelium to extracts of PV salivary tumors. This is being done to see if a sub-cellular factor is responsible for the development-supporting effect.

Methods: In part 1 above, isolated epithelium from various organ rudiments is placed within a small tumor explant and the ensemble is transferred subcutaneously to a syngeneic newborn mouse. After 12 days, the transplant is removed, serially sectioned, and examined to determine the degree of differentiation that has taken place in the normal embryonic epithelium that was placed in the tumor. Special stains are performed on some sections to determine whether basement incumbrances have been formed or whether enzymes are present in the epithelium.

In part 2 above, the T_6 chromosome of CBA/T_6 mice is used to mark the epithelial or mesenchymal components of rudiments in which tumors are to be induced. This is done by combining salivary epithelium marked with the T_6 chromosome with salivary mesenchyme not marked with the T_6 chromosome. From this combination, tumors will be induced by infecting with PV and transplanting the infected, reassembled organ rudiment to newborn C3H/Bi x CBA/T_6 F_1 hybrid hosts. The resulting tumors will be karyotyped. If tumor cells have the T_6 chromosomes in those experiments in which the epithelium was marked, but not in those experiments in which the mesenchyme was marked, then it will be concluded that the tumors originate from epithelium, rather than mesenchyme. The opposite conclusion will be reached if the converse proves true.

In part 3 above, the electron microscope is used to study the fine structural aspects of tumor cells interacting with salivary epithelium as the latter undergoes development in the tumors. Specifically, we are interested in knowing whether basement incumbrances are formed between tumor cells and normal epithelium, whether zymogen granules can be identified in the epithelium, whether virus is present in either tumor or normal cells, and what is the nature of contacts or junctions between (a) tumor cells and tumor cells; (b) tumor cells and normal epithelium; (c) normal epithelium and normal epithelium; (d) normal epithelium and normal mesenchyme.

In the time-lapse study, motion pictures of interactions between the same 4 combinations listed above will be studied. The technical aspects of this work will be done by Mr. Morgan, using a tissue culture chamber which he adapted particularly for this type of work.

In the transfilter studies, tumor or tumor extract is placed on one side of a Millipore filter, with the epithelium on the other. The epithelium is observed for growth and differentiation. In preliminary experiments, epithelium is placed directly in contact with tumor tissue, or is immersed directly in the extract of tumor tissue. Evaluation of results will be based on direct observations of living cultures and on microscopic examination of

serial sections.

Major Findings:

1. Both salivary epithelium and pancreatic epithelium undergo morphogenesis and differentiation when PV salivary tumor is used as a substitute for their normal mesenchymes. Odontogenic and lung epithelium do not undergo significant morphogenesis when these same tumors are substituted for mesenchyme. A polyoma-induced fibrosarcoma and a spontaneous myoepithelioma of parotid gland do not support development of salivary epithelium. Thus far, therefore, the only tumors that are known to have the DSE are PV-induced salivary tumors.

2. Results of the experiment using the T_6 chromosome to mark cells giving rise to PV tumors have not yet come in. The experiment is set up, and we await the appearance of tumors whose karyotype will be the deciding factor.

3. The electron microscopic study has only started. However, we already know that basement membrane is sometimes present between the normal salivary epithelium and tumor cells. Basement membrane is also present adjacent to tumor cells that are not in contact with normal epithelium. This suggests that the tumor cells are able to produce basement membrane themselves. It has also been found that a large, membrane-associated virus is budded off into glandular lumen by normal salivary epithelium supported by tumor stroma. This virus has not been classified yet, but is probably either MTV or one of the C-type leukemia viruses. The electron microscopic interpretation is being done by Dr. Banfield.

4. The tissue culture studies, including the time-lapse work, are in progress. Salivary epithelium given PV salivary tumor as a substitute for normal mesenchyme, does not undergo significant morphogenesis, although it remains alive and grows over the external surface of the tumor explant. It seems that the tumor cells are indifferent toward the normal epithelium, whereas normal epithelium and normal mesenchyme interact in such a way that the epithelium ends up inside an envelope (capsule) of mesenchyme. It will then undergo morphogenesis. It will also undergo morphogenesis if it is experimentally placed within tumor, but not if it is only placed adjacent to a small cluster of tumor cells in a culture. If the tumors arise from epithelium, then it will appear that in becoming neoplastic, the epithelial cells acquired the ability to synthesize the factor(s) which they had previously depended on mesenchyme to provide. This would account for their acquisition of independence, but it would not tell how it was brought about.

It seems rather probable that the DSE will prove to be a particulate comparable to that which has been found by Rutter in whole chick embryo extract and which supports growth and morphogenesis of pancreas, but not salivary gland. If so, one would suspect that DSE is related to some component of cell membranes, and it may be this component that is operationally responsible for the neoplastic properties of the cells.

5. The trans-filter experiments have just been started, and no results are available.

Significance: The significance of the discovery that PV-induced salivary tumor can support epithelial morphogenesis and differentiation was discussed in last year's report. The main meaning this discovery holds for research in developmental biology is that for the first time a tissue with demonstrable activity (PV salivary tumors) is available in sufficient bulk to allow extractions to be performed for concentration and identification of a chemical substance(s) responsible for the activity.

The biologic significance of this activity relevant to neoplastic properties will be more readily interpreted when the results are in from the experiment using the T_6 chromosome marker to identify the cell of origin of PV-induced salivary tumors.

Proposed Course: The experiments outlined here will be completed before planning additional ones. If the trans-filter experiments permit demonstration of activity in a sub-cellular extract or fraction, then the next step would be to find chemical methods of concentrating and identifying the active factor.

Publications:

1. Dawe, C.J., Morgan, W. D., and Slatick, M.S.: Salivary gland tumors in the role of mesenchyme during salivary gland morphogenesis. In press. Hahnemann Symposium on Epithelio-Mesenchymal Interactions. Raoul Fleischmajer (Ed.)

Serial No. NCI 526
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Carcinogenesis in the primate

Previous Serial Number: Same

Principal Investigators: Margaret G. Kelly and Roger W. O'Gara

Other Investigators: Michael D. Walker

Cooperating Units: Laboratory of Chemical Pharmacology, NCI

Man Years (July 1, 1967 - June 30, 1968)
Total: ½
Professional: ½
Other: 0

Project Description:

Objectives: (1) To study the effect on newborn and immature primates of carcinogens which have proven effective in inducing tumors in rodents. (2) To use tumor-bearing primates for studying the pharmacologic and oncolytic effects of chemotherapeutic agents as a preclinical screening.

Methods: A breeding colony of about 130 adult females is maintained, and about 80 live births are obtained per year. The newborn monkeys are mostly rhesus and cynomolgus, but a few are greens. The infant monkeys are placed on one of the following regimens at birth and maintained on it until they reach puberty: 3-methylcholanthrene (3-MC) (oral, subcutaneously or intradermal); dibenzopyrene (BP) (subcutaneously); azo dyes (oral); 2-fluorenylacetamide (2-FAA) (oral); copper chelate of N-OH-FAA (subcutaneously); urethane (orally) with and without supplementary x-radiation; diethylnitrosamine (DENA) (orally and intraperitoneal); N-nitrosopiperidine (oral); cycasin (oral); N-isopropyl-a-(2-methylhydrazino)-p-toluemide (MIH) and aflatoxin (oral). There are currently over 200 monkeys under experimental treatment.

Major Findings: There were 111 autopsies and 29 biopsies performed on experimental monkeys during the 1967-68 reporting year.

Intracerebral tumor transplants (61 autopsies and 3 biopsies)

These monkeys had received intracerebral transplants of hepatic cell carcinoma, originally induced with diethylnitrosamine, and propagated in

Benzpyrene (7 autopsies)

One Galago monkey, a prosimian primate, injected subcutaneously in the right shoulder with 3,4-Benzpyrene at birth, developed a large fibrosarcoma at the injection site. This monkey was injected by Dr. Cooper of the San Diego Zoo with carcinogen sent to him by Dr. Kelly, and housed at the zoo. The latent period was less than 26 months. Since then we have had additional Galago monkeys injected with benzpyrene, and they are now housed either at Hazelton Laboratories or at Bionetics.

Miscellaneous (3 autopsies)

One monkey treated with urethane for 48 months died of pneumonia. Two monkeys treated with Procarbazine (MIH) died, one from kidney nephrosis with hemoglobulinuria and proteinuria, and the other from cortical necrosis of kidneys.

Significance: Hepatomas have been induced in about 40 monkeys to date by the administration of DENA by oral or intraperitoneal route. Metastases have occurred in 13 of 18 autopsied cases. Alpha-fetoprotein has been found in the sera of nearly all of the monkeys bearing primary hepatomas, and in none of the monkeys without hepatomas. The presence of alpha-fetoprotein in the sera thus offers an excellent diagnostic test for hepatoma, as well as a method for evaluating any response of hepatomas to treatment.

The induction of a sarcoma in one of the lower sub-human primates suggests that these lower forms may resemble more closely the rodent in their response to polycyclic hydrocarbons than do the higher forms (rhesus, cynomolgus etc). A still lower form of primate (the tree shrew) is being studied for carcinogenic response (to benzpyrene and nitrosamine) by Dr. Richard Adamson of NCI.

Proposed Course: An attempt is being made to correlate the production of alpha-fetoprotein in tumor-bearing monkeys with the histopathology and liver function tests, by using immunodiffusion with specific anti-fetoprotein rabbit serum. Attempts are also being made to determine whether alpha-fetoprotein is produced by tumor cells in tissue culture and by transplanted tumors (intracerebral).

Treatment with carcinogens will be continued in the monkeys and it is hoped that an observation period of at least 10 years (from 1962) will give some basis for determining which carcinogens are likely to be effective in man and which are not.

Publications

1. Dawe, C.J., Whang-Peng, J., Morgan, W.D., O'Gara, R.W., and Kelly, M.G.: Culture of a cell line (NCLP-6) derived from a hepatocarcinoma induced in Macaca mulatta by N-nitroso-diethylamine. J. Nat. Cancer Inst. In Press.

tissue culture (by Dr. Dawe and later by others) for 2-3 years. Some received monkey to monkey transplants, and others were transplanted from tissue culture stock. Some of the transplants were made into newborn monkeys, but most were made into juvenile or adult monkeys with the help of immunosuppressive drugs. There were 16 tumor transplants which showed progressive intracerebral growth and 4 other tumors which grew for awhile and later regressed. A few subcutaneous or intramuscular transplants were also made in some of these monkeys, but no growth of tumor occurred in these sites.

DENA (18 autopsies and 22 biopsies)

All of the autopsied cases had hepatomas or hepatic cell carcinomas. Seven of the 18 cases also had cirrhosis of the liver while 11 did not. Thirteen monkeys showed metastases to the lungs and other organs, while 5 had no metastases. There were no animals with cirrhosis alone.

Of the 22 biopsies, 18 showed hepatoma or hepatic cell carcinomas and 4 of these also had cirrhosis. There were no animals with cirrhosis alone, although one showed only nodular hyperplasia of the liver.

Studies on the sera of monkeys for the presence of alpha-fetoprotein showed that this fetoprotein was present in the sera of 17 of 23 monkeys with hepatomas on initial examination. The sera later became positive on several of the originally negative monkeys, and only two monkeys have remained persistently negative. Sera on 69 monkeys without tumors, treated with DENA and several other liver carcinogens, have remained consistently negative.

One monkey receiving 1-Nitrosopiperidine developed a large hepatoma during this reporting period. (There were 2 autopsies and 1 biopsy on monkeys on this compound).

Aflatoxin (13 autopsies and 1 biopsy)

Many of these autopsies were done on monkeys used to determine the acute toxic dose of aflatoxin and showed only acute necrosis of the liver. However two monkeys, on lower doses, showed marked proliferative changes in the liver after 7 months of treatment. The new purified injectable aflatoxin will allow us to control the dose and toxicity better, and we believe will eventually result in liver tumors with this compound.

Cycasin (2 autopsies and 1 biopsy)

The autopsies have not yet been studied histologically.

Azo Dyes (4 autopsies)

One monkey showed marked arteritis and periarteritis of pulmonary vessels. Livers, however, show only slight toxic changes.

2. Hull, E.W., Carbone, P.P., Gitlin, D., O'Gara, R.W., and Kelly, M.G.: α-fetoprotein in monkeys with hepatomas. Proc. Am. Assoc. Cancer Res. 9: 33, 1968.

Serial No. NCI 526a
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Chemical carcinogenesis

Previous Serial Number: Same

Principal Investigator: Roger W. O'Gara

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1
Professional: 1
Other: 0

Project Description:

Objectives: (1) To study the pathogenesis of induced tumors and (2) To investigate experimentally the effect in animals of certain natural products ingested by man.

Methods: The usual pharmacologic, biologic, and histopathologic techniques.

Major Findings:

Metals

NIH black rats were implanted bilaterally with smooth or rough-surfaced iron squares, measuring 1.0 cm in diameter. Four out of 22 rats bearing rough iron squares and 4/23 rats bearing smooth iron squares developed fibrosarcomas at the implant site. However the latent period was much longer for the rough iron (23½ mos.) than for the smooth iron (18½ mos.).

Copper behaved differently than other metals, and the first two series of experiments resulted in sloughing of the copper implants in all cases from both implant sites. A third attempt, implanting copper squares deeply in the muscles of the back, showed that within 2 weeks the copper became surrounded by a large fluid-filled cyst, which persisted indefinitely. Three sarcomas have arisen to date in the walls of these cysts and the latent period has been relatively short (8, 9 and 11 months respectively).

A group of NIH black male rats have received bilateral implants of plastic discs. Half of them also subsequently received 3 or 4 large oral doses

of 3-methylcholanthrene, to determine whether a level of circulating carcinogen in the blood would accelerate the formation of sarcomas about the implants. No tumors have developed in the first six months in either group.

Significance: Previous work on metal transplants had shown the following tumor induction: for aluminum 8 tumors in 18 rats with a mean latent period of 22 months; for rough iron 1/25 tumors (latent period 21 mos.); for zinc 1/23 tumors latent period 29 mos.). Copper and aluminum remain uncorroded during their stay in the body tissues, but iron and zinc implants show marked discoloration and corrosion. The greater chemical reactivity (and consequently roughened surface) may account in part for the apparently lesser carcinogenic activity of iron and zinc in the solid state. The reason for the great accumulation of fluid about the copper implants is not known, since the surface of the copper remains bright and shining, but presumably enough copper dissolves in the tissue fluids to produce this toxic effect.

Proposed Course: No further work is planned until the results of the plastic disc implantation and MCA treatment are known.

Major Findings:

Plant studies

Aqueous extracts of 10 plants, used as a beverage or tonic on Curacao, have been assayed for carcinogenic activity, orally in mice or intramuscularly in rats. Assays on 4 other plants are almost completed, and tests on several additional plants have just started.

One fibrosarcoma was obtained from repeated injections of an aqueous solution of Krameria ixina in 15 rats. Recently we acquired dried material from aqueous and methanol extracts of Krameria (prepared by Dr. G.J. Kapodia of Howard University). This dried material was suspended in olive oil and injected into 3 groups of 15 rats each about 2 months ago. Additional rats are also being injected with an aqueous extract of Krameria prepared as before but in greater strength.

In Swiss mice 8 out of 23 developed tumors (exclusive of pulmonary tumors) after receiving Mampuritu in their drinking water for 17 months, including one large squamous carcinoma of the forestomach and an adenocarcinoma of the duodenum. In Swiss mice given Kalbas 6/24 developed leukemia or reticulum cell sarcoma. These two plant extracts (and perhaps one other) will be studied further for carcinogenic activity. Eight other groups of mice showed 1 to 6 tumors per 24 mice (exclusive of lung tumors). Results on 4 additional groups are still incomplete, and testing of 4 new herb teas has just started. Testing of combinations of Welensali (which has detergent properties) and two of the previously tested drugs is also being done, in accordance with our information that such combinations are common on Curacao.

Significance: Several of the plant extracts so far tested show suggestive evidence of low carcinogenic activity but not for the esophagus. Some of these are being repeated using a stronger brew, or using a different method of administration. If the results are repeatable, it will afford the strongest test of significance. One of the most puzzling features is the occurrence of subcutaneous sarcomas in four mice on the oral treatment (in different groups). It is difficult to dismiss them as spontaneous, since we have not previously seen spontaneous subcutaneous sarcomas in this strain of mice.

Proposed Course: We will complete the tests outlined above before making further plans.

Publications

1. O'Gara, R.W. and Brown, J.M.: Comparison of the carcinogenic actions of subcutaneous implants of iron and aluminum in rodents. J. Nat. Cancer Inst. 38: 947-957, 1967.

Serial No. NCI 526b
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Carcinogenicity of heated fat

Previous Serial Number: Same

Principal Investigator: Roger W. O'Gara

Other Investigators: Laura Stewart and W. C. Hueper

Cooperating Units: Microbiological Associates, Inc.

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/12
Professional: 1/12
Other: 0

Project Description:

A semi-final draft for publication is being prepared. This will report the results of biweekly injections into the right thigh of NIH B_1 rats (males and females) for about 17 months. (1) Non-urea adduct of fresh hydrogenated fat, 2 local sarcomas out of 35 rats (age 22 mos.). (2) Non-urea adduct of heated hydrogenated fat, 8 local sarcomas out of 34 rats (ages 11-22 mos.). (3) Non-urea adduct of fresh corn oil, 5 local sarcomas out of 44 rats (ages 7-22 mos.). (4) Non-urea adduct of heated corn oil, 16 local sarcomas out of 35 rats (ages 15-22 mos.). (5) Whole fresh corn oil - no local tumors out of 31 rats (median age 22 mos.). (6) Used whole fresh hydrogenated fat and fresh whole hydrogenated fat - no tumors out of 60 rats (median age 14 mos.). (7) Fraction A volatiles of corn oil - no local tumors out of 16 rats (median age 23 mos.).

Feeding of fats and fat fractions gave less decisive results but 2 squamous carcinomas of the forestomach arose in a group of 40 rats fed the urea-adduct of heated corn oil, and a few papillomas of the forestomach were found in all rats receiving corn oil or corn oil fractions. Three of these groups had received weekly doses of added 3,4-Benzpyrene and 3 had not. The group with the 2 carcinomas had not.

Single injections of various fats and fat fractions into 864 newborn rats induced no tumors at the injection site. However, 8 of 16 lymphosarcomas occurred in a relatively small group of 155 rats that had received an injection of non-urea adduct of hydrogenated fat or corn oil. The difference in incidence, however, is probably not statistically significant.

Serial No. NCI 527
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunocytology and Physiopathology of Cancer

Previous Serial Number: Same

Principal Investigator: Richard A. Malmgren, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: 1. To apply the fluorescent antibody technique and other immunologic methods to the study of tumor protein and antigens. 2. To study the immunologic and other factors involved in carcinogenesis.

Methods Employed: 1. The fluorescent antibody technique has been used to study various antigens associated with neoplastic cells. This requires the preparation of antisera to the appropriate antigen and tagging the antibody with fluorescein isothyocyanate. The antisera thus prepared are applied to the cells under study, either tissue imprints, tissue culture cells or frozen sections and examination of these cells in an ultraviolet microscope. The studies which have been performed during the past year using this method include:

1A. Study of surface antigens on polyoma virus transformed mouse and hamster cells with mouse antipolyoma virus or antipolyoma tumor sera. (In collaboration with Dr. Takemoto).

1B. Survey of cancer and normal human sera for adenovirus 12 "T" antibody. (In collaboration with Dr. Rabson)

1C. Examination of antimelanoma antibody in the serum of melanoma patients and some normal individuals. (In collaboration with Dr. Morton).

2. Various methods for the preparation of single cell suspensions for tumor cell transplantation were evaluated.

Major Findings: 1A. A surface antigen was demonstrated on polyoma transformed mouse and hamster cells which reacted with mouse antipolyoma virus and antipolyoma tumor sera. Normal mouse sera or sera from mice immunized with other virus tumors failed to react. Similarly, nontransformed mouse cells or SV40 virus transformed mouse and hamster cells failed to react. The surface antigen was also demonstrated in cells in the lytic cycle.

1B. Survey of 126 patients with various types of cancer and from 63 normal controls for adenovirus 12 "T" antibody revealed that 14% of the cancer patients and 11% of the normals had antibody reactive with the adenovirus 12 infected green monkey kidney cells. The types of cancer which seemed most often to be associated with positively reacting sera were brain tumors, breast cancer, and colon cancer.

1C. Autologous and hemologous sera from 27 patients with malignant melanoma and 25 normal sera have been tested for reactivity against 7 different melanomas. Antimelanoma antibodies have been demonstrated in all of the 7 patients whose sera were tested against their own melanomas. Antibodies were found in 61% of the tests of homologous melanoma sera and in 20% of the homologous normal sera. The antibody nature of the reactions was confirmed by blocking and absorption studies.

2. When trypsin, pronase, a cytosieve or syringe methods were used to reduce two different solid tumors to single cell preparations it was found that the highest number of viable cells and the least cellular debris was obtained per 100 mg of tissue from the pronase technique.

Significance: 1A. Study of the surface antigen of polyoma tumor is of importance because the current belief is that the significant antigens in tumor immunity are located on the cancer cell surface. The study of such antigens is therefore vital to ultimate hopes of applying immunotherapy to cancer. The technique also was shown to lend itself to the study of virus specific antigens in different species.

1B. Efforts to demonstrate a virus etiology for human neoplasms is fraught with difficulty. The survey of human sera for adenovirus 12 "T" antibody is of interest in that connection because it demonstrated a higher incidence of "T" antibody in the serum of patients with breast, colon and brain cancer. For a number of reasons one cannot conclude that neoplasms of these types are caused by adenovirus 12. However, further study of these types of cancer seems indicated.

1C. In considering how to study human tumor antigens with the purpose of immunotherapy or demonstration of a viral etiologic agent one continually returns to the belief that the only approach is the study of the patient's tumor with his own serum. This is the inevitable conclusion because the complexity of any other antigenic system becomes so enormous that it defies unraveling. The demonstration of antimelanoma antibody in the patient's own serum points clearly to an immunologic response on the part of the patient

which indicates the tumor to be antigenic. The possible viral nature of this antigen is suggested by similar antibody being demonstrable in other melanoma patients as well as the sera of normal individuals.

2. Many tumor transplantation studies are conduced with counted numbers of cells and often the experiment depends upon an adequate number of cells from a limited amount of tumor. It is therefore essential that the optimum yield of viable cells be obtained. Of the commonly used techniques which were tested the pronase method seemed to best meet the requirements of the ideal technique.

Proposed Course: 1. The use of the fluorescent antibody technique for the study of tumor antigens will be extended. 2. Further studies of the host cell relationship in the metastatic process will be undertaken. 3. Additional studies of the factors, particularly immunologic, involved in carcinogenesis will be carried out.

Publications:

1. Carney, P.G., and Malmgren, R.A.: Comparison of techniques for obtaining single cell suspensions from tumors. Transplantation 5: 455-458, May 1967.

2. Malmgren, R.A., Rabson, A.S., and Carney, P.G.: Survey of human cancer patient sera for adenovirus "T" antibody by immunofluorescence. Int. J. Cancer 3: 30-34, Jan. 1968.

3. Malmgren, R.A., Takemoto, K.K., and Carney, P.G.: Immunofluorescent studies of mouse and hamster cell surface antigens induced by polyoma virus. J. Nat. Cancer Inst. 40: 263-268, Feb. 1968.

4. Morton, D.L., Malmgren, R.A., Holmes, E.C., and Ketcham, A.S.: Demonstration of antibodies against human malignant melanoma by immunofluorescence. J. Nat. Cancer Inst. (in press)

Serial No. NCI 528
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Transplantable tumors in mice

Previous Serial Number: Same

Principal Investigator: Thelma B. Dunn

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 3/8
Other: 1/8

Project Description:

Objectives: An important area of cancer research is the study of tumor transplantation, because the biological behavior of neoplastic tissue is often revealed more clearly in a transplanted tumor line than in the primary.

Methods: We are now carrying 15 or more tumor lines representing 7 or more distinct tumor types. Each of these has features of particular interest:

1. Myoepitheliomas (3 lines). These tumors cause an extreme granulocytic leukemoid reaction. Tumors producing a similar effect are under study in other laboratories in an attempt to isolate the granulocyte stimulating factor.

2. A renal tumor that grows slowly and produces extreme emaciation in the host mice. Similar tumors are carried by Dr. Albert Claude in Belgium, and Miss D. Calnan at the National Cancer Institute. The emaciation appears to be specific for this type of tumor.

3. An osteogenic tumor that produces bone, and metastasizes widely. This tumor has been shipped out to other investigators.

4. Several lymphocytic tumors in different inbred strains.

5. Several lines of uterine adenocarcinomas are carried. These grow slowly and are transplanted at 6-12 month intervals. They are remarkably

organoid. These tumors are being studied and described by a visiting fellow, Dr. Nubia Munoz from Colombia. Dr. Munoz has studied the transplanted uterine tumors in groups of mice that were hormonally altered. She found that one tumor was hormonally dependent, and she has studied the results of thymidine injections in these tumors. She also observed that the uterus from newborn mice will grow for a limited time. Dr. Munoz will report her findings at the meeting of the A.A.C.R. in April.

Major Findings: Many transplantable tumors produce unexplained alterations in the host. This is especially true of slowly growing, well differentiated tumors. Various types of transplantation are now being studied and compared. These are intraocular, intrasplenic, and after contact of the tissue with necrotic substances.

Significance: We believe that more chemotherapeutic trials, and other studies should be made with these slowly growing tumors which are more like human cancers than many of the transplanted murine tumors that kill the host in a few weeks.

Proposed Course: We will continue to carry these tumors, and accumulate information on them. A paper on the uterine tumors is planned, since no published descriptions are now available. A study of the granulocytosis in myoepitheliomas has been disappointing, since no depression of granulocytopoiesis resulted from the injection of leukemoid blood or spleen.

Serial No. NCI 538
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: A study of phylogenetic aspects of neoplasia

Previous Serial Number: Same

Principal Investigator: Clyde J. Dawe

Other Investigators: J. Harshbarger, W. D. Morgan, M. S. Slatick and Charlotte Kenton

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: The major objective of this project is to perform comparative studies of neoplasia in invertebrate and lower vertebrate animals, in order to gain a comprehensive view of the biology of neoplasia throughout the various animal phyla. This is done by collecting and studying the pathologic anatomy and the natural history of neoplasms, as well as by conducting laboratory studies concerned with induction or transplantation of neoplasms in lower animals. Phenomena such as regeneration, dedifferentiation, autotomy, molting, metamorphosis, graft rejection, and so on are studied in relation to the biology of neoplastic disease. The project also includes collection of literature concerned with neoplasms of invertebrate animals.

Major Findings:

1. The Rejection Response of the Cockroach to Transplants of Tissue Culture Cells from Vertebrates. This study has been concluded and published. It was found that in spite of insects' inability to produce antibody of "classical" type, the cockroaches were able to produce an exuberant cellular response against the foreign vertebrate cells. Fish cells appeared to retain viability up to 6 days after transplantation into the cockroach hemocele. The eventual death of transplanted cells was associated with an intense hemocyte response which led to encapsulation of the transplants, with melanization of the capsule. From these findings, it was concluded that the cockroach has an immune cellular response which is apparently the chief factor in protection against heterologous tumor transplants. The response observed was very similar to that which has been described against parasites of insects.

2. Registry of Tumors of Lower Animals. The Registry continues under a contract with the Smithsonian Institution. In its second year of activity the Registry has been under the Directorship of Dr. John Harshbarger, who replaced the temporary Director, Dr. George Cantwell. Accessions increased from 76 to 123 during the year following April 1967. The accessions and activities of the Registry are reported separately in the 1967-1968 Annual Report prepared by Dr. Harshbarger.

The collection of literature on neoplasms of invertebrate and cold-blooded vertebrate animals continues, under the direction of Dr. Harshbarger and with assistance from Miss Charlotte Kenton of the National Library of Medicine.

Much time has been devoted to organization of a symposium on Neoplasms of Invertebrate and Primitive Vertebrate Animals, to be held at the Smithsonian Institution on June 19-21, 1968. This program will include papers by investigators from many foreign countries as well as the U.S.A. Reports of several new naturally-occurring and experimentally-induced neoplasms will be presented. This meeting is expected to put new thought and energy into comparative oncology; it is the first gathering of investigators of neoplasia in the most primitive phyla of the animal kingdom.

Significance: The rejection of heterologous neoplastic cells by the cockroach is not particularly surprising, but carries some fundamental significance relating to current theories that are being tested, concerning the role of immunologic mechanisms in carcinogenesis. For example, Dr. Robert Good postulates that cellular immune mechanisms, of the type represented by thymic function in vertebrates, were developed by higher animals as a "policing system" to identify and eliminate aberrant (cancer) cell population. If this is true, then it might be expected that lower animals would not have such an effective "police system" and that even heterologous tumor grafts might succeed in growing.

Since this did not prove to be the case, some alternative possibilities need to be considered: (1) Lower animals may have an effective "police system" that simply represents the fore-runner of the thymic system, phylogenetically. Such a homologue should be looked for and characterized. (2) Heterologous rejection may be too crude a test for defense mechanisms. Rejection may have been aided by the fact that the chemical environment of the cockroach hemocele is not optimal for replication of vertebrate animal cells. Therefore, a more meaningful experiment would be to test the cellular response of cockroaches to neoplastic insect cells, of homologous and isologous origin. (3) Some other mechanisms unlike anything known in higher animals may be operative in the lower animals to protect them against neoplastic populations. These should be looked for, as they might, theoretically at least, be useful in designing ways of protecting higher animals against neoplasms.

Proposed Course: No further studies of transplantation of vertebrate animal cells into cockroaches are planned. It is believed that the logical step now is to see whether continuous cell culture lines derived from insect

tissues can grow in homologous or isologous hosts. At this moment, we are not experienced in growing insect cells, and are not properly equipped for this type of investigation. Insect tissue culture specialists are in a better position to advance this field, but if they do not follow this line of research, we may go into it. No experimental work will actually be started until the symposium has been held; this will permit a better appaisal the state of research in this area.

Publications:

Dawe, C. J., Morgan, W. D., and Slatick, M.S.: Cellular response of a cockroach Leucophaea madera to transplants of cell culture lines of vertebrates. Fed. Proc. 26: 1698-1706, 1967.

Serial No. NCI 557
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Review of reticulum cell sarcoma, Type B, (Hodgkin's like lesion) in various inbred strains of mice.

Previous Serial Number: Same

Principal Investigator: Thelma B. Dunn

Other Investigators: Margaret K. Deringer

Cooperating Units:

Man Years (July 1, 1967 - June 30, 1968)
Total: 0
Professional: 0
Other: 0

Project Description:

Objectives: A large group of reticulum cell neoplasms is found in the mouse which resembles Hodgkin's disease in man. Lesions of this type were described in a publication by Dunn in 1954, but much more information has accumulated since then. Since these are perhaps the most confusing neoplasms in mice, another publication has been prepared to appear in the April issue of the JNCI.

Methods: Dr. Deringer had available large collections of reticulum cell neoplasms in old mice of inbred strains BALB/c, C57BL and C57L. A review of these tumors, and of publications on similar tumors since 1954 has been completed.

Major Findings: These tumors show a wide range of morphologic variation, they are difficult to diagnose, and similarity to human Hodgkin's disease is debatable.

Significance: An animal counterpart of Hodgkin's disease would be a useful experimental model, and a better understanding of the disease in mice might throw light on some problems relating to the human disease.

Proposed Course: See project No. 566, a study of transplanted Type B neoplasms.

Serial No. NCI 558
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Maintenance of mice on a liquid diet, and long term administration of an anti-fertility substance (Enovid) to mice

Previous Serial Number: Same

Principal Investigator: Thelma B. Dunn

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 3/8
Professional: 1/4
Other: 1/8

Project Description:

Objectives: To determine the feasibility of long continued liquid diet for mice and the incorporation of various substances. Anti-fertility substances are now widely used in human populations, but there is little information on the effect if given to animals for a long period of time at a low dose.

Methods: Enovid was administered in a powdered mash diet for over a year at 4 times the calculated human dose. This method of dosing mice was very unsatisfactory, no results were observed, so incorporation in a liquid diet (Metrecal) has been tried.

Major Findings: The mice have now been on the Enovid for nearly two years and only a few have been autopsied. Mice on Metrecal alone do well, but there appears to be a decline in fertility.

Significance: A safe, reliable contraceptive method must be found if the "population explosion" is to be controlled. However, any substance proposed for human use should be tested for a long time in animals. Since these substances produce a hormonal imbalance, they are potentially carcinogenic and may have other serious effects.

Proposed Course: Mice now on Enovid will be autopsied when they appear in poor health. Mice on Metrecal will be kept through a number of generations. A few have been started on whole milk to see whether this will maintain them.

Serial No. NCI 560
1. Laboratory of Pathology
2. Cancer Ind. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Electron probe and scanning electron microscope

Previous Serial Number: Same

Principal Investigator: William G. Banfield

Other Investigators: Biodynamics Research Corporation

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: 1. To learn how and in what areas the electron probe and scanning electron microscope can be applied to biological research.

2. To become proficient in the use of these instruments and the interpretation of data from them.

3. To keep abreast of the development of these instruments and make contributions to their development as applied to biology.

Major Findings: A continuing contract has been obtained with Biodynamics Research Corporation, Inc., to test and develop the electron probe for biological applications. The most noteworthy developments are the improved morphology using back scatter electrons with beam currents in the nanoamp range, the availability of a secondary electron detector, and the obtaining of 800A resolution using a newly-developed transmission scanning assembly with a new solid state detector.

Significance: A rapid progress in improving tissue morphology, as seen in the electron probe, is being made. This is essential for interpretation of results from elemental analysis.

Proposed Course: 1. Using a new freeze-drying method, test the applicability of the probe to elemental analysis of thyroid with particular attention to iodine. Using the same method, analyze skin for phosphorus, sulfur, iron, chlorine, potassium, sodium and calcium and compare this with other forms of fixation and embedding, paying particular attention to migration and leaching of elements.

2. Analyze asbestos bodies in two more cases of asbestosis as unknowns and compare with the supposedly known answer in the hands of Dr. Silikoff.

3. Complete titanium observations on lungs and prepare for publication.

4. Complete analysis of scleral plaques for publication.

5. Continue with a study of electrolytes, Ca and P in developing bone.

6. Complete a study of intracellular calcification in retinoblastomas.

7. Continue to improve the Biodynamics Corporation electron probe for biological applications as rapidly as possible.

8. Attempt to extend the observations on skin to viral lesions and pathological conditions as soon as feasible.

9. Set up an electron probe laboratory within NIH as soon as possible.

10. Attempt to expand Biodynamics contract commitment among other Institutes, if possible, to make for a more efficient use of the available technology and more rapid development of the electron probe.

11. Explore approaches to developing a training facility for electron probe analysis and scanning electron microscopy.

12. Consider a scanning electron microscope laboratory within NIH.

13. Continue to explore the application of x-ray and electron diffraction to problems of pathology and medical research in general.

Serial No. NCI 561
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Collaborative research and other activities

Previous Serial Number: Same

Principal Investigator: Mearl F. Stanton

Other Investigators: None

Cooperating Units: SVCP, SALES, JNCI

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/2
Other: 0

Project Description: (Other Activities)

Fifty percent of the normal work week is spent as Scientific Editor of the JNCI. Ten percent of the normal work week is spent in administrative activities related to contracts with the Special Virus Cancer Program, Comparative Pathology Contract with the Smithsonian Institution, and long-term animal holding facility contract with Melpar, Inc.

Proposed Course: These activities will probably continue if I remain in the good graces of the Director, NCI and the contributors to the Journal. In addition, it is anticipated that some time will be devoted to the establishment of a new electron microscopy laboratory and a laboratory for the study of lower forms of animal life. As I write this during the late hours of the night, relation to the normal work week loses its meaning.

Serial No. NCI 562
1. Laboratory of Pathology
2. Cancer Induction and Pathogenesis Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Continuous culture of a liver tumor from a rhesus monkey

Previous Serial Number: Same

Principal Investigator: Clyde J. Dawe

Other Investigators: W. D. Morgan, Jacqueline Whang-Peng, Edgar W. Hull, Margaret G. Kelly, Roger W. O'Gara

Cooperating Units: Laboratory of Chemical Pharmacology, NCI
Medicine Branch, NCI

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/2
Other: 0

Project Description:

This project is a continuation of a study that has now extended over more than 3 1/2 years. During that period of time a cell line (NCLP-6) derived from a nitrosamine-induced liver tumor of a rhesus monkey has been grown continuously in culture. Details of the origin and characteristics of the tumor are given in last year's report and in J. Nat. Cancer Inst. 39: 153-169, 1967. The advantages this cell line offers for cancer research are:

1. It is useful for comparative studies of liver cancer, because it is the only existing cell line of liver cancer from a subhuman primate. Several cell lines exist that are derived from liver cancers of rodents.

2. The cultures cells can grow into tumors that kill the host, when transplanted intracerebrally into homologous (rhesus) animals.

Major Findings:

1. The original culture line was distinctly epithelium-like in its morphology, and after 70 subcultures this property has been retained in some sublines. However, on 3 separate occasions fibroblast-like variants appeared, which tended to displace the epithelial population in the vessels in which the variants arose. These variants are now carried as subline designated "F" (fibroblast-like), as opposed to "E" (epithelium-like sublines).

2. Karyotype studies of the E subline and one F subline show that both are subtriploid. The modal number for the E subline is 59, while that for the F subline is 56. Both sublines have at least one small acrocentric marker chromosome and one large metacentric marker chromosome. A constant difference between the 2 sublines is the presence of an extra #20 chromosome in the E subline, while the F subline remains disomic for this chromosome.

3. The E subline by cytochemical tests shows large amounts of granular alkaline phosphatase in the cytoplasm, while the F subline has almost none.

4. The E subline, when allowed to remain in a culture vessel without frequent subculture, undergoes a simple type of morphogenesis. Micropolyps and micropapillae are formed, and the epithelium on the outside of cell masses forms a palisaded covering layer. This is done in the absence of any stromal cells.

F subline cells form no definite structures, but tend to arrange themselves in small "schools" of parallel spindle cells whose long axes are perpendicular to the long axes of spindle cells in the neighboring "schools".

5. Dr. Hull has found, by the Ouchterlony plate method, that culture fluids from the E subline contain alpha-fetoglobulin, an antigen that was found by Abelev and associates to be present in murine and human liver cancers. The fluids from cultures of the F subline gave negative reactions for alpha-fetoglobulin.

Significance:

1. Because of its transplantability, this cell line is of use in studies of chemotherapy of liver tumors. Since it grows in the brain of recipients, it may also be used for studies of the pathologic effects of intracranial neoplasms, and for blood-brain barrier studies.

2. In culture, the line can be used for study of cell metabolism, in comparison with normal liver cells. Such studies have already been made by Westfall, Pitot, Thompson, Tomkins, and others, using rodent liver tumor cultures.

3. It provides a primate liver cell that can be used for virus propagation. It should be of interest to investigators of human infections, hepatitis, in particular.

4. Morphologically the E subline is of interest because of its autonomous morphogenetic activities. This offers an opportunity to study mechanisms of simple structure formation at the cell level. The line is also of interest because of its tendency to throw off spindle-cell variants. This phenomenon is of interest to pathologists, who are familiar with sarcoma-like variants of carcinomas in vivo.

5. The E subline may be especially useful for production and purification of alpha-fetoglobulin. This would lead to the preparation of better anti-sera for clinical testing, as well as to basic studies of the normal function and structure of alpha-fetoglobulin.

Proposed Course: Cells and culture fluids are being provided to Dr. Hull for his studies of alpha-fetoglobulin. If his need for these materials increases greatly, it may be expedient to have a contractor produce the cells.

The cell line is available to other investigators on request. It will be offered to the Cell Culture Type Collection for storage and distribution.

A report characterizing the NCLP-6 cells has been accepted for publication in the JNCI.

Publications:

1. Kelly, M.G., O'Gara, R.W., Walker, M.D., Dawe, C. J., Morgan, W. D., and Kerber, W. T.: Intracerebral transplantation into rhesus monkeys of cell cultures (NCLP-6) derived from a chemically induced primate hepatocarcinoma. J. Nat. Cancer Inst. 39: 153-169, 1967.

2. Dawe, C. J., Whang-Peng, J., Morgan, W.D., O'Gara, R.W. and Kelly, M. G.: Culture of a cell line (NCLP-6) derived from a hepatocarcinoma induced in Macaca mulatta by N-nitroso-diethylamine. J. Nat. Cancer Inst. In press.

Serial No. NCI 565
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Effect of granulocytosis on tumor growth

Previous Serial Number: Same

Principal Investigator: Thelma B. Dunn

Other Investigators: Barbara Lei

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 0
Professional: 0
Other: 0

Project Description:

Objectives: To test the possible inhibiting effect of granulocytosis on the growth of a transplantable fibrosarcoma in comparison with a granulocytic leukemia.

Methods: BALB/c mice were transplanted with a myoepithelioma which produces an extreme leukemoid granulocytic reaction, and later transplanted with a fibrosarcoma at 6 different intervals to see whether the growth is affected. Using the same myoepithelioma, Dr. Arthur Upton of the Biological Laboratory of the Atomic Energy Commission at Oak Ridge tested the effect of granulocytosis on a granulocytic leukemia.

Major Findings: No difference was detected between growth of fibrosarcoma and granulocytic leukemia.

Significance: Some have postulated that the growth of a neoplastic blood cell might be inhibited by the normal blood cell counterpart which sends back the message that no more cells of its type are needed (Osgood theory). We had an indication that the erythroblastosis caused by the Rauscher virus in BALB/c mice can be inhibited by transfusion. The present experiment failed to show any inhibitory effect.

Proposed Course: Since the granulocytic neoplasm was not inhibited by the myoepithelioma, no further work is planned.

Serial No. NCI 566
1. Laboratory of Pathology
2. Cancer Induc. & Pathogenesis
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: A study of reticulum cell sarcoma, Type B, or the Hodgkin's like lesion in BALB/c mice.

Previous Serial Number: Same

Principal Investigator: Thelma B. Dunn

Other Investigators: Barbara Lei

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 0.1
Professional: 0.1
Other: 0

Project Description:

Objectives: To obtain more precise information on this neoplasm, which is frequently found in old BALB/c mice. At the present time, information on this neoplasm is insufficient.

Methods: A large group of old untreated BALB/c mice, and those that were on Enovid chow meal have become available for autopsy. Very complete autopsies are done, and suspicious lesions are transplanted. Some transplants will be made to the spleen.

Major Findings: Several tumors have been successfully transplanted, and carried for several generations. One tumor line has changed to a lymphocytic neoplasm.

Significance: Tumors of this type are now described in SJL/J and NZB mice, and it is speculated that an immunity process is involved in their pathogenesis. The neoplasms appear in about 20 percent of old BALB/c mice, and the conditions are less complex than in these other strains.

Proposed Course: To continue over a period of time until a significant mass of data is accumulated.

Serial No. NCI 501
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Geographic pathology field studies on urinary bladder cancer in New Orleans, Louisiana

Previous Serial Number: Same

Principal Investigators: Lucia J. Dunham, Harold L. Stewart, and Harold F. Dorn (deceased)

Other Investigator: Alan S. Rabson

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: The objective of this project is to complete an extensive questionnaire study of patients with urinary bladder cancer in a southern U.S.A. city with a high incidence of bladder cancer compared to two other southern cities. The data have been analyzed with a view to identifying factors associated with cases of urinary bladder cancer. Basic data on the incidence and pathologic (microscopic) diagnosis of the cancers were obtained.

Methods: For the area under study and the period of study all patients possible with the diagnosis of urinary bladder cancer were interviewed, and an equal number of control patients as well. The data recorded included residence, occupation, medical and surgical history, and information about diet and about the use of alcoholic and non-alcoholic beverages and of tobacco. Records were kept of the small number of patients not interviewed, their diagnoses, and the reasons for failure to interview.

A medical social worker interviewer was employed in the project through March 31, 1964. A coding statistician assisted part-time throughout the study from February 1, 1958. The total period of study was 6 years.

Major Findings: Data from 493 patients with cancer and 527 control patients have been reviewed and coded. Tables have been prepared. Diagnostic tissues have been reviewed from all cancer patients who were interviewed, and also from 147 patients not interviewed.

Significance: The significance of the study lies in the hope of supplying a sound scientific basis for the understanding of factors in habit or environment as they may be related to a high incidence of urinary bladder cancer.

Proposed Course: The report has been submitted to the Editorial Board of the Journal of the National Cancer Institute.

Serial No. NCI 501a
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Geographic pathology field studies on uterine cancer in New York City, Israel, and Washington, D. C.

Previous Serial Number: Same

Principal Investigators: Harold L. Stewart, Lucia J. Dunham, Louis B. Thomas, and Harold F. Dorn (deceased)

Other Investigator: John H. Edgcomb

Cooperating Units: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/2
Professional: 1/2
Other: 0

Project Description:

Objectives: The objective of this project was to complete an extensive questionnaire study of women with uterine cancers in different geographic areas and racial groups, and to analyze the data obtained with a view to identifying factors suspected of predisposing to or causing female genital cancers. Also, basic data on the incidence and pathologic (microscopic) diagnoses of the cancers were obtained.

Methods: For the area under study and the period of study all patients possible with the diagnosis of uterine cancer were interviewed and an equal number of control patients as well. The confidential data recorded include social, medical, and surgical history, and menstrual, marital, and pregnancy data. Records were kept of the small number of patients not interviewed, their diagnoses, and the reasons for failure to interview. Pathologic material was studied in all cases.

Major Findings: 1. Epidemiologic Study of Cancer of the Uterus in Jewish and Non-Jewish White Women. 2. Epidemiologic Study of Cancer of the Uterus in Negro Women. a) In New York City. Data from 458 patients with cancer of the uterus and 530 control patients have been reviewed and coded. Tabulation and analysis of data are complete. Pathologic material has been examined in the cancer cases. There are small differences in the environmental factors of patients with cancer of the cervix and control patients. Most of these are not statistically significant. b) In Washington, D. C.

Data from 363 patients with cancer of the uterus and 380 control patients have been reviewed and coded. Cancer of the uterus in Negro women will be compared in the two cities.

Significance: The significance of the study lies in the hope of supplying a sound scientific basis for the understanding of factors of custom, habit, or environment as they may be related to relatively high frequencies of uterine cancers in some groups of women as contrasted to intermediate or relatively low frequencies in other groups. In addition, a critical evaluation of the study, prepared at the same time as the report, should provide a useful commentary on techniques in the epidemiology of cancer.

Proposed Course: A report of the study of Jews and Non-Jews in New York City and Israel was published in July 1966. A report of the study of Negro patients in New York City and Washington, D. C. is being prepared for publication.

Publications:

1. Dunham, Lucia J.: Epidemiology of Tumours of the Uterine Cervix. Europ. J. Cancer 3: 491-495, 1968.

Serial No. NCI 501b
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Epithelial atypia in hamster cheek pouches treated repeatedly with calcium hydroxide.

Previous Serial Number: Same

Principal Investigators: Lucia J. Dunham, Calum S. Muir, James E. Hamner

Other Investigators: None

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Objectives: To ascertain the effects on the hamster cheek pouch of factors added to treatment with calcium hydroxide, i.e. treatments with other substances, and modification of the environment supplied by the test animal.

Methods: The right cheek pouches of 70 hamsters in 6 experimental groups are being treated with calcium hydroxide powder five times each week. The groups are (1) fed with three types of deficient diet or offered drinking water containing 20% alcohol, (2) hamsters with their salivary gland secretion reduced by chemical means, (3) hamsters treated with calcium hydroxide in the morning and several types of foreign protein in the afternoon, (4) hamsters treated with calcium hydroxide in the morning and a finely divided silicate in the afternoon, (5) hamsters treated with calcium hydroxide in the morning and 6 types of plant material in the afternoon.

New techniques of testing the above substances are being explored.

Major Findings: The experiments are in progress and there are not yet any major findings.

Significance: The lesions of epithelial atypia resembled dyskeratosis (leukoplakia) in man except that they were focal, and did not widely involve the cheek pouch in any instance. Oral cancers are relatively frequent in betel quid chewers. The relationship of these cancers to the lime or calcium hydroxide that was added to the quids is uncertain. Perhaps damage such as is produced in the hamster pouch by repeated application of calcium hydroxide renders the affected area more susceptible to the effects of a carcinogen. It was attempted to find out whether other treatment or modification of the

environment would cause progression of the epithelial atypia to cancer.

Proposed Course: A report was published in 1966. The present investigation will be continued; when completed a report will be prepared for publication. A review of the literature pertinent to relationships of tissue injury and carcinogenesis is planned.

Publications:

1. The Relationship between Oral Cancer and Plants--a Geographic Study. To be published in a U.I.C.C. Monograph reporting the proceedings of a Symposium on Carcinogens of Plant Origin, to be held April 8-10, 1968 in Bethesda, Maryland.

Serial No. NCI 501c
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Effects of gambier, an ingredient of the betel quid, administered to mice by various routes

Previous Serial Number: Same

Principal Investigators: Calum S. Muir and Lucia J. Dunham

Dr. Muir has the semifinal draft of the report.

Serial No. NCI 501d
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Attempt to induce tumors by injecting newborn mice with pollutants from processed water

Previous Serial Number: Same

Principal Investigators: Lucia J. Dunham, Roger W. O'Gara and Floyd B. Taylor

Other Investigators: None

Cooperating Units: PHS-Environmental Engineering and Food Protection Division

Man Years (July 1, 1967 - June 30, 1968)
Total: 0
Professional: 0
Other: 0

Project Description:

This project has been published.

Publications:

1. Dunham, L.J., O'Gara, R.W. and Taylor, F.B.: Studies on pollutants from processed water. Collection from three stations and biologic testing for toxicity and carcinogenesis. Amer. J. Publ. Health, 57: 2178-2185, 1967.

Serial No. NCI 501f
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Maps - Geographic Pathology of Cancer

Previous Serial Number: Same

Principal Investigators: Lucia J. Dunham and John C. Bailar

Other Investigators: None

Cooperating Units: Demography Section, Biometry Branch, NCI

Man Years (July 1, 1967 - June 30, 1968)
Total: 1/4
Professional: 1/4
Other: 0

Project Description:

Objectives: To prepare for publication maps that show mortality rates of cancers at about 8 sites in men and 8 sites in women. An explanatory text, selected incidence and relatively frequency data on cancer, and references to the source materials will accompany the maps.

Methods: Published reference material has been analyzed and selected by the 2 authors and the data standardized. Several investigators have generously contributed unpublished data.

Major Findings: Sixteen maps and sixteen tables of standardized data have been prepared. The accompanying text includes a discussion of the maps. Several interesting patterns of cancer distribution are apparent from study of these materials.

Significance: The significance is to present a world-wide collection of data on the distribution of cancers in such a way that it will facilitate studies in geographic pathology.

Proposed Course: Maps, tables, and accompanying text will be published in the Journal of the National Cancer Institute in July 1968.

Serial No. NCI 501g
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Report on histologically diagnosed cancers in Indians of the United States, 1950 to 1966

Previous Serial Number: None

Principal Investigators: Gert L. Laqueur, John C. Bailar and Lucia J. Dunham

Dr. Laqueur has the semifinal draft of the report.

Serial No. NCI 531
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: In vitro studies of human malignant lymphomas

Previous Serial Number: Same

Principal Investigator: Alan S. Rabson, M.D.

Other Investigators: Richard A. Malmgren, M.D., Philip M. Grimley, M.D., and Elizabeth W. Chu, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968).

Total: 1
Professional: 2/3
Other: 1/3

Project Description:

Objectives: To study some biological properties of human malignant lymphoma cells in vitro.

Methods Employed: Standard tissue culture, virologic and pathologic techniques are used.

Major Findings: Another new line of lymphoblasts (AL-4) has been established from an American patient with a "Burkitt tumor" and is being compared with our previous African and American lines. It contains small numbers of the herpes-like EB virus and supports the growth of herpes simplex.

In collaboration with Dr. Victor Bedoya, a visiting scientist from Colombia, and Dr. Grimley, we have been studying growth of herpes simplex virus in lymphoma cells in vitro. The virus grows well in these cells with characteristic cytopathic effects and some interesting changes in cell membranes. We have been carefully studying the biological properties of herpes simplex after sequential passage in lymphoma cells but thus far have been unable to identify any significant differences from the same strains passed in other cell systems.

We have also been attempting to isolate "focus-forming" viruses from the lymphoma cells and from human sarcomas maintained in vitro using a variety of human cell strains as indicator systems. Thus far all of these attempts have been unsuccessful.

With the possibility that some human cytomegaloviruses may be involved in etiology of human lymphomas, we have been studying the properties of some of the known strains of this group. They are difficult to work with and grow slowly, but we have finally worked out good methods for growth and assay. With Dr. Gelderman, we have studied the interaction of toxoplasma and human cytomegalovirus in vitro.

In collaboration with Dr. Mary Ann Guggenheim, an NCI Postdoctoral fellow in our laboratory, we have attempted to apply the Sendai virus cell fusion techniques of Henry Harris to the recovery of latent viral genomes from human lymphoma cells but these studies have thus far been unsuccessful. In collaboration with Dr. Friedman, we have applied the cell fusion techniques to the study of interferon production in heterokaryons produced from chicken red blood cells and human cells. Chicken interferon was produced when the avian nucleus was within a human cell which can itself produce human interferon but not when it was fused into a human cell which cannot produce human interferon. The results open the way for a series of important experiments on control of interferon production.

Significance: The importance of the search for possible viral etiology of human malignant lymphomas is evident. In addition, any information about the biological properties of human lymphoma cells may be of importance in our understanding of the lymphoma problem.

Proposed Course: Attempts to isolate viruses from human lymphomas and sarcomas will continue using both focus-forming assay systems and attempts to "rescue" viral genetic material by passage of other animal viruses in the human malignant cells.

Publications:

1. Guggenheim, M.A., Friedman, R.M., and Rabson, A.S.: Interferon: Production by chick erythrocytes activated by cell fusion. Science 159: 542-543, Feb. 1968.

Serial No. NCI 532
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of SV40 and Oncogenic Human Adenoviruses in vitro and in vivo

Previous Serial Number: Same

Principal Investigator: Alan S. Rabson, M.D.

Other Investigators: Richard A. Malmgren, M.D., Ruth L. Kirschstein, M.D., David Paulson, M.D., Daniel Albert, M.D., Leonard Deftos, M.D., and William Strohl, Ph.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives: To study some biological properties of primate viruses with oncogenic activity in Syrian hamsters.

Methods Employed: Standard virologic and pathologic methods have been used.

Major Findings: A. With Dr. Paulson and Dr. Fraley (NCI-Surgery), we have been studying transformation of hamster prostatic tissues. We have produced transformed cell lines and tumors derived from prostate which contain significant amounts of tartrate-inhibitable ("Prostatic") acid phosphatase. We are now studying effects of steroid hormones on growth and biochemical properties of these cells.

B. With Dr. Albert (Ophthalmology Branch-NINDB) we have been studying in vitro transformation of ocular tissues. Explant cultures of adult hamster retina, choroid, and iris were infected with four oncogenic DNA viruses: (1) Simian virus 40 (SV40); (2) the LLE46 strain of adenovirus 7; (3) polyoma virus; and (4) human adenovirus 12. Most cultures underwent transformation as evidenced by sustained rapid growth, development of an acid pH soon after instillation of fresh media, and formation of multiple layers of cells and clumps. After several in vitro passages, transformed cells injected subcutaneously into irradiated adult hamsters produced tumors at the

injection sites.

All ocular tissue transformed with SV40 and produced neoplasms composed of epithelioid and spindle-shaped cells with large nuclei; however, no pigmented tumors were observed. Choroid and iris transformed with LLE46 produced tumors histologically similar to the SV40 type growths. Retina infected with LLE46 produced tumors which were bimorphic, containing in addition to the SV40-like component, areas of small hyperchromatic cells resembling an adenovirus-induced tumor. Polyoma virus transformed only retina, producing tumors with elongated cells with hyperchromatic pleomorphic nuclei and small amounts of basophilic cytoplasm. Adenovirus 12 also transformed only retina and gave rise to typical adenovirus-type neoplasms composed of layers of small, darkly-staining cells. Although these tumors had some resemblance to retinoblastomas, no rosettes or neural elements could be identified.

C. With Dr. Brown (NCI-Surgery) we have been continuing studies of transformation of pineal glands. Transformed lines of monkey pineal tissue have been produced and are now being studied for enzyme content and ability to produce tumors in homologous hosts.

D. With Dr. L. Deftos of NHI we have been studying the effects of SV40 transformation on parathyroid tissues in vitro. Cells from a human parathyroid adenoma were infected with Simian virus 40 and maintained for successive generations in monolayer tissue culture. These "transformed" cells produced parathyroid hormone, identified by radioimmunoassay and density gradient ultracentrifugation, through thirteen generations for over nine months of growth. Studies of effects of calcium concentrations on production of parathyroid hormone are in progress.

E. In collaboration with Drs. William Strohl and Harriet Rouse, we have studied the role of the adenovirus 12 genome in determining the morphology of adenovirus-type tumors. Adenovirus type 12 transforms the fibroblastic BHK21 (baby hamster kidney) cell line into rounded or cuboidal cells that give rise in hamsters to undifferentiated small cell sarcomas indistinguishable from those induced in newborn hamsters by inoculation of the virus itself. In contrast, cells from this line transformed by polyoma virus retain their fibroblastic morphology and induce fibrosarcomas in hamsters. This suggests that the morphology ot tumors induced by the adenovirus-transformed cells from this line may be determined by the viral genome and that such a mechanism may also explain the remarkably uniform microscopic appearance which seems to characterize tumors induced in hamsters by direct inoculation of adenovirus type 12.

Significance: The studies of transformed endocrine tissues again indicate that it is possible to incorporate a viral genome into a functional endocrine cell and still retain some specific function. The studies with prostatic tissue suggest that such transformed cells may also retain sensitivity to steroid hormones. The studies of the effects of adenovirus transformation on the BHK21 cells indicate that in some cases the morphology

of a tumor may be more dependent upon the etiologic agent than upon the tissue of origin. This suggests that traditional histopathologic criteria for determining histogenesis of tumors may at times be inappropriate.

Proposed Course: Studies to determine effects of exogenous stimuli on parathyroid hormone production in vitro and the effects of steroids on the transformed prostatic tissue will be continued. We will also continue our studies of the role of the viral genome in the induction of transformation and the possible role of the oncogenic DNA viruses in human cancer.

Publications:

1. Strohl, W.A., Rabson, A.S., and Rouse, H.: Adenovirus tumorigenesis: Role of the viral genome in determining tumor morphology. Science 156: 1631-1633, June 1967.

2. Deftos, L.J., Rabson, A.S., Buckle, R.M., Auerbach, G.D., Potts, J.T.: Parathyroid hormonal production in vitro by human parathyroid cells transformed by SV40. Science 159: 435-436, Jan. 1968.

3. Paulson, D.F., Rabson, A.S., and Fraley, E.E.: Viral neoplastic transformation of hamster prostate tissue in vitro. Science 159: 200-201, Jan. 1968.

4. Malmgren, R.A., Rabson, A.S., and Carney, P.G.: Survey of human cancer patient sera for adenovirus "T" antibody by immunofluorescence. Int. J. Cancer 3: 30-34, Jan. 1968.

5. Guggenheim, M.A., Friedman, R.A., and Rabson, A.S.: Interferon: Production by chick erythrocytes activated by cell fusion. Science 159: 542-543, Feb. 1968.

6. DiPaolo, J.A., Rabson, A.S., and Malmgren, R.A.: In vitro viral transformation of chemically transformed cells. J. Nat. Cancer Inst. (in press)

7. Orme, S.K., Wells, S.A., Jr., and Rabson, A.S.: In vitro neoplastic transformation of hamster pineal tissue by oncogenic DNA viruses. Cancer (in press)

Serial No. NCI 536
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: 1. A review of the surgical and autopsy sections from veterans of World War I whose cause of death has been listed as lung cancer.

2. Collaborative work with Dr. G. Wynne Griffith, Pan American Health Organization.

3. Collaborative work with members of Panel on Mesotheliomas

Previous Serial Number: Same

Principal Investigator: Katherine McD. Herrold

Other Investigators: Harold L. Stewart; Members of the Panel on Mesotheliomas: Chairman: Dr. Jacob Churg; Members: Drs. Harold L. Stewart, Samuel H. Rosen, Werner A. Laquer, Roland Guy, Desmond E.O. Magner, Sylvan Moalten, Bernard Naylor, Gerritt W.H. Schepers and Russel B. Sherwin.

Cooperating Units:

Man Years
Total: ¼
Professional: ¼
Other: 0

Project Description:

Objectives: The objective of the study is to review all the cases from veterans of World War I which have been diagnosed as lung cancer and to determine whether the findings meet the criteria for primary lung cancer. The second objective is to classify the tumor according to the histological type.

Methods: This review is being done in conjunction with the epidemiological study of the National Cancer Institute on the relationship between smoking and death from carcinoma of the lung.

Major Findings: A manuscript entitled "A Survey of the Histological Types of Primary Lung Cancer in U.S. Veterans, World War I" was submitted to Cancer during February 1968. Histological material from 1477 cases of lung cancer was reviewed. The diagnosis of primary lung cancer was confirmed in 70.9%; equivocal in 23.9%; not primary lung cancer in 4.7%; not a case of cancer in 0.5%. The frequency of the histological types was determined. No correlation was found between the histological type and cigarette smoking. In the non-smokers Group I tumors, epidermoid and oat cell carcinomas comprised 27.3% of the 55 cases reviewed. The ratio of Group I to Group II tumors is primarily dependent on the source of material examined.

Passey has stated that one can say without hesitation that the age at death from lung cancer is certainly not that which we associate with chemical carcinogenesis. The results of the present study lend support to Passey's view because in this series the age at death from lung cancer was not related to the age started to smoke, the number of years smoked or the number of cigarettes smoked per day.

Significance: The statistical data concerning smoking stresses that a high percentage of lung cancer cases are smokers. Extremely important from a biological standpoint is that only a small percent of heavy cigarette smokers develop lung cancer. In the Dorn study of smoking, only 9.9% of the heavy smokers (2 or more packs of cigarettes/day) had lung cancer: 90.1% who were heavy smokers did not develop lung cancer but died from other causes.

If cigarette smoking is one of the etiological causes of epidermoid and oat cell carcinoma and not of adenocarcinoma, then one should not find Group I tumors in non-smokers. In the present study Group I tumors were found in non-smokers, and the occupation of the individuals was not associated with any increased risk.

There is clearly a need for uniform agreement in the precise classification and terminology of primary lung cancer. Until such agreement is reached and the nomenclature standardized it will be impossible to assess the relationships of histological types to prognosis, survival time and evaluate the results of various forms of therapy. If statistical results are to be compared it is imperative that there be a wide acceptance and usage of the same rules for classification of primary lung cancer. Kreyberg's classification is comprehensible and reproducible. It is hoped that his classification will be adopted and that the term oat cell is retained.

Proposed Course: Material from some of the lung cancer deaths, included in the Inter-American Investigation of Mortality, Pan American Health Organization project, is being reviewed. Primary lung cancer cases from La Plata, South America and Bristol, England are being classified according to histological type, using Kreyberg's method.

Some of the plans formulated by the Mesothelioma Panel include:

1) Organization of regional panels in North America.

2) In order to collect material, an attempt will be made to give publicity at pathology meetings both national and regional.

3) Determination of the rate of mesothelioma at the present time by examining all available cases of diffuse tumors of the pleura and peritoneum in the hospitals of two or three of the largest cities in the United States.

4) Preparation and study of submitted material and circularization to the members of the panel.

5) Special forms for epidemiological and pathological data will be available.

6) Set up criteria for the histological diagnosis of mesothelioma.

Publications:

1. Herrold, K.M.: A survey of the histological types of primary lung cancer in U.S. Veterans, World War I. Submitted for publication February 1968, Cancer.

Serial No. NCI 536a
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Experimental studies relating to the carcinogenic activity and the mode of action of the nitrosamine compounds.

Previous Serial Number: Same

Principal Investigator: Katherine McD. Herrold

Other Investigators: None

Cooperating Units:

Man Years
Total: ¼
Professional: ¼
Other: 0

Project Description:

Objectives: The objective of this study is to determine what factors influence the localization of tumors induced with the nitrosamine compounds. From a biological standpoint to attempt to determine the mechanism of the carcinogenic action of diethylnitrosamine, N-nitroso-methylurethane and N-nitroso-methylurea.

It has been reported in the literature that certain of nitrosamine compounds induce primary brain tumors including astrocytomas, oligodendrogliomas and sarcomas. The objective of some experimental studies now in progress is to determine if the nitrosamines pass the blood-brain barrier.

Methods: Syrian hamsters are being used as the experimental laboratory animals. The following routes of administration of diethylnitrosamine have been used. 1) Intragastric; 2) Subcutaneous; 3) Intratracheal; 4) Intraperitoneal; 5) Intradermal; 6) Local or skin painting; 7) Subcutaneous and intragastric administration to prepare animals to determine if DENA or a carcinogenic metabolite passes the placenta; 8) Subcutaneous and intragastric administration, postnatally, to determine if DENA or carcinogenic metabolite is excreted via the milk; 9) Intravenous administration of certain of the nitrosamines. The dose of the carcinogen and interval period between treatment is being varied.

The routes of administration with N-nitroso-methyl-urethane include: 1) Subcutaneous; 2) Intragastric; 3) To pregnant animals; 4) Postnatally.

The routes of administration used with N-nitroso-methylurea include intravenous, intragastric, intraperitoneal, subcutaneous and intratracheal.

Major Findings: (1) All Syrian hamsters that were administered N-nitroso-N-methylurethane, intragastrically, developed epidermoid carcinomas of the forestomach which were locally invasive. Thirteen of the 16 animals had epidermoid carcinomas of the esophagus. A few of the animals revealed thecomas of the ovary, papillomas of the vagina, and stromal hyperplasia of the endometrium. (2) Subcutaneous administration of N-methyl-N-nitrosourea induced malignant undifferentiated subcutaneous sarcomas in all animals at the injection site. Most of the animals had metastases either in the lung or lymph nodes. (3) Pigmentary degeneration of the retina, odontogenic tumors and epidermoid carcinomas of the oral cavity; malignant tumors of the esophagus, forestomach, intestine and colon are induced by N-nitroso-methylurea. (4) Tumors of the breast, ovary, uterus and vagina are also induced with N-nitroso-methylurea. (5) The histogenesis of malignant liver tumors induced by dimethylnitrosamine (DMN) was studied in Syrian hamsters. The observed series of lesions that started from simple proliferation of reticuloendothelial cells developed into tumors that had the characteristic histological picture of hemangioendothelial sarcomas with multiple lung metastases. The sequential series of lesions that started out as simple bile-duct hyperplasia eventuated in cholangiofibrosis. Cholangiofibrosis appears to be the usual precursor for the development of cholangiocarcinomas. No tumors of hepatic cell origin were observed. The concept that all induced malignant tumors of the liver in laboratory animals arise from hepatic cells is not valid for Syrian hamsters. (6) Odontogenic tumors and epidermoid carcinomas of the oral cavity were induced in Syrian hamsters by N-nitroso-N-methylurea (NMU). The induced odontogenic tumors showed a striking histologic similarity to the human counterpart of this type of tumor. The epidermoid carcinomas were often multicentric and locally invasive. The site of origin included the gingiva, buccal mucosa, palate and tongue.

Significance: The results of these experimental studies have demonstrated that the localization of tumors induced by some of the nitrosamine compounds is the same regardless of the route of administration. The possible relevance to gastrointestinal cancer in man is that carcinomas of the esophagus, stomach and intestine may result from a carcinogen that enters the body via a portal of entry other than the oral route.

The findings also suggest that N-nitroso-N-methylurea or a metabolite, in addition to the carcinogenic action, interferes with specific metabolic processes as evidenced by the retinotoxic effect and disturbances in odontogenesis. Degeneration of the retina following administration of a drug ranks high among nonfatal clinical disasters. This experimental model of induced pigmentary degeneration of the retina in Syrian hamsters by NMU may not only provide another tool for studying retinal function but may serve as a technique for screening drugs, which are to be used in man, to determine evidence of retinal toxicity.

Faulty odontogenesis is considered to be the basis for the development of odontogenic tumors in man. Valuable clues to the solution of physiological and metabolic problems are waiting to be discovered in the teeth of experimental animals. The teeth of rodents are valuable biological indicators which during their development mirror and record the metabolic status of the animal. This experimental model of induced odontogenic tumors and associated disturbances in odontogenesis may provide not only a means for studying the histopathogenesis of these unusual tumors but also a method for determining whether certain types, such as an ameloblastic fibroma, mature into a compound odontoma.

Proposed Course: (1) Continue observations and analyses of data from the different experimental groups under section on methods.

Publications:

1. Herrold, K.M.: Epidermoid carcinomas of esophagus and forestomach induced in Syrian hamsters by N-nitroso-N-methylurethane. J. Nat. Cancer Inst. 37: 389-394, 1966.

2. Herrold, K.M.: Carcinogenic effect of N-methyl-N-nitrosourea administered subcutaneously to Syrian hamsters. J. Path. & Bact. 92: 35-41, 1966.

3. Herrold, K.M.: Histogenesis of malignant liver tumors induced in Syrian hamsters by dimethylnitrosamine. J. Nat. Cancer Inst. 39: 1099-1111, 1967.

4. Herrold, K.M.: Pigmentary degeneration of the retina induced by N-methyl-N-nitrosourea. An experimental study in Syrian hamsters. Arch. Ophth. 78: 650-653, 1967.

5. Herrold, K.M.: Odontogenic tumors and epidermoid carcinomas of the oral cavity. An experimental study in Syrian hamsters. Oral Surg. Oral Med. Oral Path. 25: 262-272, 1968.

Serial No. NCI 536b
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: Experimental studies relating to the induction of lung tumors in Syrian hamsters.

Previous Serial Number: Same

Principal Investigator: Katherine McD. Herrold

Other Investigators: None

Cooperating Units:

Man Years
Total: ¼
Professional: ¼
Other: 0

Project Description:

Objectives: The objective of the study is the induction of lung tumors in the Syrian hamster and to determine the early changes in the bronchial epithelium that may be precancerous lesions. Attempts are also being made to determine if certain hormones or other substances influence the histologic type of primary lung cancer induced.

Methods: Syrian hamsters are being used as the experimental laboratory animals. Intratracheal instillation of test substances is performed with a modified Pasteur pipette (TePunga's technique). The laboratory animals are divided into the following experimental groups: 1) Benzo(a)pyrene dissolved in olive oil; 2) Benzo(a)pyrene suspended in distilled water; 3) Benzo(a)-pyrene suspended in Tween 60; 4) Thorium dioxide colloidal solution; 5) Cigarette smoke condensate suspended in Tween 60; 6) Atmospheric pollutants and atmospheric extracts suspended in Tween 60; 7) Benzo(a)pyrene suspended in Tween 60 and additional treatment with hormones; 8) To study the similarity or differences between lung tumors induced by chemical carcinogens and those induced in the lungs of Syrian hamsters by the Polyoma-agent. The experimental model was the injection of tumor inoculum intravenously. The effect of N-nitroso-methylurethane administered subcutaneously to Syrian hamsters has been studied. The effect of other nitrosamine compounds on the respiratory system of Syrian hamsters is also being studied.

Major Findings: A wide spectrum of pathological changes were observed in the lungs of the experimental animals that received N-nitroso-methylurethane subcutaneously. These included: PAS-positive proteinaceous material in the alveolar spaces, acicular clefts, prominent epithelialization of the alveolar walls, masses of desquamated cells in the lumina, pulmonary corpora amylacae, cystic spaces and intense interstitial fibrosis. The atypical acinar proliferation was characterized by air spaces lined with mucus secreting bronchiolar type of epithelium pulmonary adenomatosis and irregular shaped cystic spaces lined by anaplastic cells in peripheral areas of the lung associated with dense scarring.

Significance: N-nitroso-N-methylurethane, a known potent carcinogen, is widely used in chemistry for the preparation of diazomethase, an alkylating agent. The above findings support the concept that environmental agents that reach the lungs by routes other than inhalation may be relevant to the problem of not only lung cancer in man but also to other obscure pulmonary diseases such as pulmonary alveolar proteinasis, desquamative interstitial pneumonia, and chronic interstitial fibrosis. The search for chemicals, drugs, or other substances that have a selective affinity for pulmonary epithelium may further our understanding of lung cancer and other pulmonary diseases in which the alveolar membrane is altered.

Proposed Course: Continue observations and analysis of data from the different experimental groups under section on methods.

Publications:

1. Herrold, K.M.: Fibrosing alveolitis and atypical proliferative lesions of the lung. An experimental study in Syrian hamsters. J. Path. 50: 639-651, 1967.

Serial No. NCI 536c
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Project Title: 1. Comparative pathological study of gastric carcinoma in Japanese migrants.

2. Experimental gastrointestinal carcinogenesis in Syrian hamsters.

Previous Serial Number: Same

Principal Investigators: Katherine McD. Herrold

Other Investigators: Harold L. Stewart, John Berg, Biometry Branch, NCI; Kaneyoshi Akazaki, Director of Aichi Cancer Center Research Institute, Nagoya, Japan; Prof. Nobuaki Sasano, Tohoku University, Sendai, Japan; Dr. Grant Stemmermann, Honolulu, Hawaii.

Cooperating Units:

Man Years
Total: ¼
Professional: ¼
Other: 0

Project Description:

Objectives: The objective of the study is to review the pathologic material obtained from patients with gastric cancer. Interests include the gross features and location of the cancer within the stomach, and possible association with ulcer, adenomatous polyps and intestinal metaplasia in relation to the pathogenesis of this type of cancer.

Methods: It is anticipated that a large volume of material will be reviewed and the findings will be correlated with the epidemiological study conducted by Mr. William Haenszel and Dr. Segi.

In addition to the gross and microscopic study of the bases of stomach cancer other aspects include:

a) Virus Study: This part of the study is being performed in collaboration with Dr. Ray Bryan, Chief, Viral Oncology. Specimens of gastric cancer from cases in Hawaii and Honolulu will be investigated for the presence of virus.

b) Electron Microscopic Study: A contract has been signed with Tohoku University School of Medicine, Sendai, Japan, for electron microscopic examination of stomach cancer. The main objective is to determine the presence or absence of virus particles in the tumor tissue.

A preliminary report of the electron microscopic findings of the cases of gastric cancer so far studied was presented at a combined meeting of pathologists and epidemiologists. This meeting was held in Honolulu, Hawaii during November 1964. No definite virus particles have so far been identified. Intracellular particles were observed and believed to represent aggregations of RNA, glycogen, ferritin and proteins. The identity of others has not been determined.

Some of the findings suggest that by electron microscopic examinations of gastric cancer it will be possible to determine the cell type that gives rise to the tumor. In cases of adenocarcinoma, the tumor cells resembled foveolar epithelium, whereas tumors of the so-called signet ring type suggested origin from chief cells.

c) Histochemistry: The different types of mucin will be determined in the cases of stomach cancer by histochemical methods. The results of this study are at present incomplete. Preliminary studies with N-nitroso-methylurea administered subcutaneously to Syrian hamsters has revealed alterations in the mucosa of the glandular stomach. The glandular hyperplasia reveals a striking alteration in the proportion of cell types with almost total absence of chief and parietal cells. The mucous in the focal areas of gastric epithelial hyperplasia has shown a distinct change in staining properties comparable to that which occurs in intestinal metaplasia in man.

Dr. Grant Stemmermann, Director of Laboratories, Kuakini Hospital, Honolulu, Hawaii, in addition to the histochemical studies being performed to characterize the type of mucin present in cases of stomach cancer, is doing extensive enzyme studies. He presented a preliminary report entitled "Gastric Mucins" at the IX International Cancer Congress, Tokyo, Japan. Subsequently he published this material in the Journal of the National Cancer Institute.

Stemmermann, G.N.: Comparative study of histochemical patterns in non-neoplastic and neoplastic gastric epithelium: A study of Japanese in Hawaii. J. Nat. Cancer Inst. 39: 375-382, 1967.

d) A conference was held in Los Angeles, California during January 1966. Those attending included Dr. John Berg, NIH; Dr. John Dunn, Chronic Diseases, Department of Public Health, Berkeley, California; Dr. Milton Rosenthal and Dr. Meyer Zeiler, Culver City, California; Dr. John H. Yoell, Beverly Hills, California and Dr. Katherine McD. Herrold, NCI. Plans were formulated to collect pathological material from cases of stomach cancer among Japanese residing in California.

e) A conference of the pathologists and epidemiologists was held in Honolulu, Hawaii during April 1967.

Significance: A study of this type in different parts of the world might disclose the correlation between the pathologic findings and the epidemiological findings pointing towards possible etiologic factors. It may also enhance knowledge of the pathogenesis of gastric cancer.

Proposed Course: The following are the proposed projects for future studies of gastric cancer in Japanese migrants and in native Japanese:

I. Study of Sub-Sample.

1. Review of 100 cases from Japan and 100 cases from Hawaii.
2. Blocks of tumor to be re-cut and stained with H & E. Code number assigned to each case.
3. The purpose is to determine if there is a difference in frequency involvement for both groups, using a double blind technique, for the following:
   a) Signet ring cells at different levels of invasion of stomach wall.
   b) Stroma. Two low power microscopic fields to be examined and evaluated for $\leq 50\%$ stroma and $> 50\%$ stroma.

II. Diagramatic or Grid Plotting of Gross Location and Size of Tumors. (Suggested that this work be done at Tohoku University, Sendai, Japan)
1. Size of tumor.
2. Location of T tumors.

III. Re-code, carcinoma in situ listed in cases from Hawaii as mucosal carcinoma.

IV. Ulcer, suggested means of coding.
A. Separate from tumor.
B. Adjacent to tumor.
C. Completely surrounds tumor.

V. Collect 100 cases of gastric ulcer and 100 grossly normal stomachs, from medical examiner cases for comparative study. Purpose is to get a sampling and determine where the shift is.

VI. Histochemistry and Ultrastructure.

Attempt to correlate histochemistry with ultrastructure. Determine the substance in the chief cells which stains as an acid mucosubstance.
1. Comparative study of histochemistry and ultrastructure of gastrointestinal spontaneous and induced tumors in laboratory animals and man.
2. Comparative ultrastructural study of large intestinal carcinoma.

3. Extend the ultrastructural studies to include studies on gastric mucosa of fetuses, newborns and infants and also the regenerating epithelium at ulcer margins.
4. Expand the ultrastructural study at present operating in Japan to include:
   a) Studies on early cases of gastric cancer.
   b) Studies of intestinal carcinoma and intestinal metaplasia. (Japanese definition of early stomach cancer includes cases where invasion of tumor is limited to the mucosa and submucosa).

VII. Immunochemical.
1. Attempt to characterize differences between neoplastic and non-neoplastic nucleic acids. Nucleoprotein chemist would be essential for the study.

VIII. Japanese Histological Classification of Stomach Cancer (Page 8-Pathology Protocol).
1. Suggested that Japanese code cases of stomach cancer from Hawaii according to this method of histological classification.

IX. Histological Classification of Cases of Stomach Cancer Related to Level of Involvement.
1. Mucosa.
2. Submucosa.
3. Muscle coat.
4. Serosa.
5. Metastases.

X. Collect 100 cases of gastric ulcer cases both in Japan and Hawaii. Purpose is to evaluate non-neoplastic changes and tabulate findings according to page 11 of the stomach cancer pathology protocol.

XI. Page 11 of the Gastric Cancer Pathology Protocol.
1. Items to be coded and correlated with age, sex and other factors.
2. Suggested that mucosal strips be used instead of swiss roll technique.
   The following sections to be taken:
   a) antrum; b) lesser curvature;
   c) junction; and d) body

XII. Clinical Studies.
1. Glucose tolerance tests and insulin measurements. Purpose would be to correlate with presence and degree of intestinal metaplasia in cases of gastric ulcer and gastric cancer.
2. Determination of circulating antibodies.

XIII. Histochemistry on Gross Specimens (Stomach).
1. Alkaline phosphate on excised stomachs in order to map out the distribution of intestinal metaplasia.

Serial No. NCI 555
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ultrastructural and Radioautographic Studies in Arbovirus Virus Infection

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Robert M. Friedman, M.D. and Irene K. Berezesky, B.S.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives: To correlate morphological changes in infected cells during arbovirus synthesis with biochemical studies.

Major Findings: In a system utilizing Semliki Forest virus in chicken fibroblasts, unique structures thought to represent centers of viral synethsis and/or assembly have been demonstrated in the cytoplasm by electron microscopy. The apparent mechanism of viral entry and release from the cell has also been demonstrated.

Significance: The significance of such investigations lies in the stated objective: Results contribute basic information to virology and molecular biology.

Proposed Course: In timed sequential studies and utilizing a variety of electron microscopic techniques including radioautography it is hoped that a morphological demonstration, which correlates with the biochemical information, can be made of the intracellular events which occur after arbovirus infection.

Publications:

1. Friedman, R.M., and Berezesky, I.K.: Cytoplasmic fractions associated with Semliki Forest Virus ribonucleic acid replication. J. Virology 1: 374-383, April 1967.

Serial No. NCI 563
1. Laboratory of Pathology
2. Cancer Pathology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of mechanisms of action in tumor immunology

Previous Serial Number: Same

Principal Investigator: Costan W. Berard, M.D.

Other Investigators: Richard A. Malmgren, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 3/4
Professional: 1/4
Other: 1/2

Project Description:

Objectives: 1. To study the significance of serologic factors in tumor immunity.

2. To study the significance of cellular factors in tumor immunity.

3. To employ techniques of (1) and (2), where possible, in the study of human tumors.

Methods Employed: 1. For the study of "serologic immunity", the principal methods are fluoresence microscopy and isotope-release cytotoxicity. The former is used to detect the presence of cell-bound antibody, whether or not the union is damaging to the cell. The latter technique employs chromium-51, used to "tag" the target cells; release of the isotope as a consequence of antigen-antibody-complement union provides a quantitatible index of cell damage. Complement fixation methods are also used to quantitate cellular antigenicity.

2. For the study of "cellular immunity", standard tissue culture techniques have been used for the maintenance of tumor cells and immuno-competent cells in vitro. Tritium-labeled thymidine and/or uridine were used to study cellular replication and interaction.

3. A "double-label" technique, utilizing fluorescent-antibody and radioautography on single coverslip preparations of cultured cells, is used to relate antigenicity to cellular metabolic events, e.g. presence and quantity of SV40 "T" antigen at various phases of the mitotic cycle.

4. Standard histopathologic techniques are used, where applicable, to assist in interpretation and evaluation of in vivo immune mechanisms.

Major Findings: 1. "Serologic Immunity":

No evidence of serologic immunity has been found in mice with chemically-induced tumors by the techniques employed in these studies. In the hamster-SV40 system, however, antibody to "T" is demonstrable in suitably immunized animals. The quantity of antigen in tumor cells, however, varies with time in the same cultured line. This variation seems to be related to cellular rates of multiplication, cell density, and metabolic state.

2. "Cellular Immunity":

In studies of chemically-induced solid tumors of mice, the basis of immunity has been previously shown to depend in vivo upon action of immunocompetent cells (spleen, lymph node, peritoneal lining cells). Adoptive (passive) transfer of immunity from an immune to a susceptible animal has been demonstrated when such cells were used for the transfer. To investigate the mechanism of action of immunocompetent cells upon tumor cells, an in vitro test system was developed:

Using enzymatic methods, a technique for preparing viable, rapidly dividing single-cell suspensions of methylcholanthrene-induced fibrosarcomas was perfected. As a source of immunocompetent cells, peritoneal lining cells were selected. Of all immunocompetent cells studied, they had the highest survival rate in tissue culture. Furthermore, since the donor animal was little affected by the procedure of peritoneal washing, the same animal was used for serial studies. The cells of both types were studied in mixed cultures for 96 hours duration.

Completed studies have shown that tumor cells in vitro are significantly but inconstantly affected by peritoneal cells from an immunized, in comparison to a control, animal. Instead of the target-cell lysis common in serologic immunity, the mechanism in cellular immunity seemed to be one resulting in inhibition of replication of the neoplastic cells. The growth-inhibition, however, was weak in these syngeneic systems and, unlike that in allogeneic experiments, was not reproducible enough to parallel studies in vivo.

Significance: The tissue-culture system developed in this study can be used to investigate the mechanisms operative in cellular immunity. Heretofore, such immunity has been extensively studied in vivo but such studies were limited with regard to defining mechanisms of action. Only in vitro, for example, has it been possible to show that the principal effect is

one of growth-inhibition rather than target cell-lysis. It has become apparent, however, that the reproducible effects on target cells reported in allogeneic mixed-cell systems are much more difficult to demonstrate in syngeneic systems. If an approach is to be made in vitro to the quest for human tumor antigens, one must anticipate that the factors involved will be most similar to those in syngeneic murine systems and thus may be subtle and inconstantly demonstrable in vitro.

Proposed Course: Since syngeneic immunity was not reproducibly demonstrable in vitro in chemically-induced murine tumors, these efforts have been temporarily suspended. Work is at present directed to studying "T" antigens of virus-transformed cells in vitro, with the hope that cellular and serologic immunity may be more approachable in this experimental model. If quantitative, reproducible results can be achieved, efforts will be made to study human tumors by similar techniques.

Serial No. NCI 517
1. Laboratory of Pathology
2. Pathological Technology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1967 - June 30, 1968

Part A:

Project Title: Pathological Technology Section

Principal Investigator: Joseph M. Albrecht

Other Investigators:

Cooperating Units:

Man Years (July 1, 1967 - June 30, 1968)
Total: 30
Professional: 4
Other: 26

Project Description:

Stained tissue sections are the fundamental basis of all clinical and experimental studies of cancer. The Section prepares histological sections for all the investigators of the National Cancer Institute. It makes available all the established routine and special stains and in addition develops and provides the current experimental methods of tissue preparation such as enzyme stains and specific histological stains.

July 1, 1967 - June 30, 1968

Number of investigators	95
Number of pieces of tissue	112,945
Number of bottles of tissue	21,325
Number of blocks cut	71,514
Number of blocks cut serially	2,306
Number of frozen blocks cut	525
Number of blocks recut	1,536
Number of slides stained H & E	97,823
Number of slides stained special	19,094
Number of unstained slides	10,044

Contract Work - Baker Histology Laboratory

Number of bottles of tissue	1,966
Number of pieces of tissue	21,424
Number of blocks cut serially	14
Number of slides stained H & E	12,177
Number of slides unstained	91

Photographic Service rendered to the Laboratory of Pathology, July 1, 1967 - June 30, 1968.

Gross
- Black & White..... 215
- Color..... 26

Micros
- Black & White..... 1282
- Color..... 690

Lantern Slides
- Black & White..... 198
- Color..... 361

Prints
- 4 x 5..... 3165
- 8 x 10..... 405
- Mounted..... 404

Part B included: No

Serial No. NCI 852
1. Pathologic Anatomy Branch
2. Cytopathology Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Exfoliative cytology applied to human diagnostic problems and research problems

Previous Serial Number: Same

Principal Investigator: Richard A. Malmgren, M.D.

Other Investigators: Elizabeth W. Chu, M.D. and Costan W. Berard, M.D.*

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 7
Professional: 6
Other: 1

*During this period Dr. Berard of the Surgical Pathology and Postmortem Service actively participated in the functions of the Service.

Project Description:

Objectives: The primary responsibility of the Cytopathology Service is to provide the staff of the Clinical Center with an accurate and complete exfoliative cytology service. In addition, the Cytopathology Service collaborates in various clinical research projects evaluating cancer therapy, the hormonal status of the cancer patient, the course and natural history of the cancer lesion, and the anatomical and physiological changes in the human body associated with various pathologic conditions. The accomplishment of these objectives necessitates the continual improvement of techniques and the origination of new cytologic methods in the laboratory.

The specific objectives include: (1) An attempt to gain a better understanding of the factors involved in the metastasis and recurrence of human cancer associated with the surgical treatment of cancer. (2) An evaluation of the spinal fluid in leukemia to determine the value of cytology in demonstrating central nervous system involvement in the leukemic process. (3) Improve the techniques used in the study of sex chromatin preparations from the buccal mucosa. (4) Setting up cytogenetic techniques which may be used for general cytology or special research problems. (5) Assessment of a new cancer detection test (in collaboration with Dr. Thomas Dutcher and Dr. John Bennett, CC, CP). (6) Reevaluation of the exfoliative cytology methods

used for hormone evaluation (in collaboration with Dr. John Marshall, NCI).

Methods Employed: The standard Papanicolaou staining procedure is applied to preparations of various human specimens. These specimens are then screened and evaluated for morphologic changes in the cellular elements. Special procedures involving the use of fluorescent microscopy, microspectrophotometry, millipore filtration, and various special staining and centrifuge techniques are required in many instances.

Patient Material:

The figures compiled below are for the last twelve months.

Type of Material Examined	Accessions	Smears
Vaginal and Cervical	1,203	2,236
Other - (Ascitic-pleural-gastric-prostatic-urine-bronchial washing-sputa-spinal fluid-sex chromatin study-wound washings-special procedures-etc.)	1,966	4,361
Total	3,169	6,597

Institutes	Accessions	Smears
NCI	2,112	4,411
NHI	303	656
NIAMD	377	664
NIAID	95	199
NINDB	173	367
NIMH	50	95
NIDR	23	45
NICHD	-----	-----
Referred	36	160
TOTAL	3,169	6,597

Major Findings:

1. The major contribution of the Cytopathology Service to the diagnostic facilities of the clinical staff has been the early diagnosis of neoplasms of the cervix and lung and of other sites not previously diagnosed. In several instances, exfoliative cytology provided the only tissue confirmation of neoplastic disease before death or treatment of the patient.

2. The second major contribution of the Cytopathology Service is to the research programs of the NIH which require cytologic methods.

3. The evaluation of wound washings, submitted by the Surgery Branch, NCI has been continued with the demonstration of neoplastic cells in many of the washings.

4. In collaboration with the Leukemia Service, NCI, examination of spinal fluid specimens from patients with leukemia using the millipore filter technique has demonstrated leukemic cells in the spinal fluid. The correlation between these observations and the presence of central nervous system involvement has been studied. In several instances the presence of a few immature white cells in the spinal fluid anticipated the development of central nervous system leukemia.

5. The standard deviation for sex chromatin determination performed in our laboratory has been made and the relationship of these determinations to hormonal status of the invididual failed to show a correlation.

6. Methods for cytogenetic studies are now established so that the Cytopathology Service can provide this service on a limited routine basis for Clinical Center studies.

7. Morphologic changes in the peripheral blood leukocytes of patients with cancer were shown statistically to exist but were not sufficiently reliable in our hands to be used as a detection test for cancer.

8. Hormone evaluation by exfoliative cytology was not found to be as reliable as other methods for determining when ovulation had occurred in patients with sterility problems.

Significance:

1. The diagnostic value of exfoliative cytology in the clinical evaluation of cancer and its early detection is readily apparent from the activities of the Cytopathology Service in the Clinical Center and throughout the country. In a number of instances unsuspected in situ carcinoma of the cervix has been detected in Clinical Center patients by the routine examination of vaginal and cervical smears on new hospital admission. Exfoliative cytology has often been the only tissue evidence of cancer in a number of patients prior to treatment or death.

2. Since the cytologic demonstration of leukemic cells in the spinal fluid has been shown to correlate with the clinical course and autopsy findings, its use as a method for assessing the need for and value of intrathecal chemotherapy make it a valuable method in leukemia management.

3. The thought has been prevalent that the numerical volume of sex chromatin counts may be related to hormonal levels of the individual. Careful statistical analysis of the data collected on repeated sex chromatin counts on individuals failed to show any significant or characteristic relationship with the hormone levels. Thus numerical values for sex chromatin counts cannot be used as simple methods for hormone assessment.

4. The need for more cytogenetic studies on a service as well as research basis has existed for some time and now is available.

5. It is of interest that cancer patients can be separated from normals on the basis of morphologic changes in their peripheral blood leukocytes. The fact that this cannot be done at a level which makes the method useful for cancer detection is unfortunate but does not invalidate the notion that one of the quantitative alterations in the tumor bearing human is morphologic changes in his peripheral blood leukocytes.

6. Many clinicians are of the opinion that vaginal cytology is a suitable means for following the ovulatory cycle. To a limited extent this is true but certain other methods appear to be more useful and complete reliance on cytology seems undesirable.

Proposed Course: The continuation of a complete and adequate cytopathology service for the staff of the Clinical Center is our primary purpose. Continued collaboration with the Clinical Center staff in various research projects and the development of new and improved techniques within the laboratory are an important part of the future program. Particular emphasis will be placed on cytogenetic studies, evaluation of the new cancer detection test and the cytologic characterization of viral infected cells and cells in mycosis fungoides patients.

The cytologic evaluation of leukemic cells in spinal fluids will be continued to further determine the value of cytology in determining the course and effect of therapy of CNS leukemia.

Publications:

1. Malmgren, R.A.: Studies of circulating cancer cells in cancer patients. In Pierre Denoix (Ed.): Proceedings of UICC Symposium on the Mechanisms of Invasion in Cancer, Paris, France July 5-10, 1965. Heidelberg, Springer-Verlag, 1967, pp. 108-117.

2. Malmgren, R.A.: Circulating cancer cells and their significance - A reappraisal. The University of Texas M.D. Anderson Hospital and Tumor

Institute 21st annual symposium on Fundamental Cancer Research. (in press)

3. Fisher, J.C., Ketcham, A.S., Hume, R.B., and Malmgren, R.A.: Significance of cancer cells in operative wounds. Amer. J. Surg. 114: 514-519, Oct. 1967.

4. Chu, E.W., Malmgren, R.A., and Kazam, E.: Variability of sex chromatin counts. Acta Cytol. (in press).

5. Malmgren, R.A., Bennett, J.M., DelVecchio, P.R., DeWitt, S.H., Feld, M., Schneiderman, M.H., and Kazam, E.: Evaluation studies of peripheral blood leucocyte changes in malignancy. Acta Cytol. (in press)

Serial No. NCI 854
1. Pathologic Anatomy Department
2. Cytopathology Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Experimental studies on carcinogenesis, endocrine factors and cytogenetic characters

Previous Serial Number: Same

Principal Investigator: Elizabeth W. Chu, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 1
Professional: 1
Other: None

Project Description:

Objectives:

1. To study the pathogenesis of lesions of rodent organs after treatment with chemical and biological agents and also to study the influence of these agents on the development of neoplasm and metastasis.

2. To study the actions of therapeutic agents on the normal epithelia (Myleran - Bisulfan)

3. To study the cytogenetic characteristics of a) long term human malignant lymphoma cells in vitro; and b) patients with premature ovarian failure.

4. To study the actions of virus either alone or in combination with podophyllin in the production of cervical lesions, possibly leading to neoplasm.

Methods Employed:

1. A. Hamsters were fed with a mixture of ground purina chow containing:
   a) 0.05% Bp
   b) 0.05% Bp plus 5% Vit. A
   c) 0.05% Bp plus 1.25% Vit. A

B. Hamsters were given:
aa) 2.5 mg. DMBA by pipette 2/week. Tap water to drink
ab) 2.5 mg. DMBA, 2/week 5% alcohol as drinking water
ac) 2.5 mg. DMBA, 2/week 1% alcohol to drink

ba) 0.05% Bp in ground food. Tap water to drink
bb) 0.05% Bp in ground food. 5% alcohol to drink
bc) 0.05% Bp in ground food. 1% alcohol to drink

Collaborator: Dr. R. A. Malmgren

2. Female hamsters are fed with regular purina chow but use: a) tap water as drinking water; b) 5% Myleran in tap water as drinking water and c) 2.5% propylene glycol in tap water as drinking water. Periodic leucocytic counts and differential counts are done using blood from orbital veins. Weekly vaginal smears are obtained and examined for any epithelial changes. (Collaborator: Dr. R. A. Malmgren)

3a. Human lymphoma cells were carried in vitro in both infected and non-infected Simian-virus. Chromosomal analyses were done on these cells at random intervals. (Collaborator: Dr. A. S. Rabson)

3b. Patients with premature ovarian failure may reveal chromosomal aberrations. (Collaborator: Dr. J. Marshall)

4a. Hamsters were immunized against Herpes virus by three intramuscular injections of the virus (1:100 dilution) at weekly intervals; after which, their cervices were painted with the same virus once and again three months later. Vaginal smears were examined at periodic intervals for pathological lesions. (Collaborator: Dr. A. S. Rabson)

4b. The cervices of young adult Syrian hamsters were painted with a 10% alcohol-podophyllin twice a week for five weeks; after which the cervices were painted with Herpes virus (1:10 dilution) once. Podophyllin paintings were again resumed. Vaginal smears were taken once a week to determine any cervical changes.

Major Findings:

1a. The incidence rate of hydrocarbon induced gastrointestinal cancer in hamsters is significantly influenced by the use of specific amounts of vitamin A. The pilot study shows that when:

Bp (0.05% in ground food) - 100% in 14 months
Bp + Vit. A (1.25%) - 71%
Bp + Vit. A (5%) - 30%

Comparable results were also seen when DMBA was used in lieu of Bp.

1b. The group of animals treated with 0.05% Bp in their food and used tap water as drinking water did not show any cancer in eight months; but the group that had the same carcinogen but used 5% alcohol as drinking water instead of plain tap water developed tumors of forestomach (40%). In another group of animals receiving DMBA plus tap water developed tumors of forestomach as well as those using 5% alcohol as drinking water.

2. The hamsters receiving 5% Myleran in their daily drinking water have not yet shown any pathological lesions; both the leucocytic picture and the vaginal smears show no abnormal conditions.

3a. The marker chromosomes seen in the primary tumor of one malignant lymphoma were not found in its long term tissue culture; yet the marker chromosomes of another case persisted in its long term culture.

3b. Leucocytic cultures of peripheral blood of one of our patients revealed 47 chromosomes with trisomy X.

4a. The hamsters that were immunized with herpes virus and later had their cervices painted developed severe cervicitis but recovered from it two to four weeks later. Subsequent paintings with the virus did not cause any recurrence of the cervicitis.

4b. Painting of the hamster cervices with podophyllin and podophyllin-herpes virus induced severe dysplasia of the cervical vaginal epithelium but no neoplastic lesions 9 months after the initial treatment.

Significance:

1a. The inhibitory effect of Vitamin A on the development of gastro-intestinal tract tumors could shed some light on the carcinogenesis of gastrointestinal cancers in humans. The present available data indicate a definite dose relationship of the Vitamin A used and tumor growth.

1b. A definite relationship of alcohol and Bp is seen in the induction of tumors of forestomach in hamsters that the ingestion of 8 months of Bp-food did not induce any tumors but tumors of the forestomach were seen in those animals that had been using 5% alcohol instead of tap water as drinking water. This has practical implications in the case of humans.

2. Dysplastic epithelial changes of bronchial and cervical epithelia in patients being treated with Myleran have been reported. It, therefore, is important to find out if the dysplasia will progress to neoplasia. The study thus far has not shown changes similar to those observed in the human; whether this tolerance of Myleran by hamsters is a specie-specific or due to other factors is not determined at present.

3. The significance of the loss of marker chromosomes in one of the cases of long term tissue culture needs to be investigated.

4. The role that can be played by a pathogenic virus in the induction of cancer needs to be thoroughly investigated.

Proposed Course:

1. Work continues on the study of effects of vitamin A on chemically induced tumors with special interest in its dose relationship. Likewise, work continues on the tumor promoting effect of alcohol on the induction of gastric neoplasia.

2. Work continues on the tumor promoting possibilities of a pathogenic virus, Herpes virus.

3. Continue to study the effects of Myleran on bronchial and cervical epithelia in hamsters.

4. Continued work on the chromosomal characters of long term tissue cultures.

5. Work will be started to study chromosomal characters and/or differences between primary and metastatic tumors in both humans and animals.

Publications:

1. Wurtman, R.J., Axelrod, J., Chu, E.W., Heller, A., and Moore, R.Y.: Medial forebrain bundle lesion: Blockade of effects of light on rat gonads and pineal. Endocrinology 81: 509-514, Sept., 1967.

Serial No. NCI 853
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Department of Pathologic Anatomy (Pathologic Anatomy Branch, NCI) has as its principal duty the responsibility to serve in investigative efforts of the N.I.H. staff responsible for patient care. In many instances, investigative study of pathologic material is of great importance to clinical research.

Staff:

* Dr. Harold L. Stewart, Chief, Pathologic Anatomy Branch
* Dr. Louis B. Thomas, Head, Surgical Pathology and Postmortem Service
* Dr. Richard A. Malmgren, Head, Cytopathology Service
* Dr. Alan S. Rabson, Pathologic Anatomy Branch
* Dr. Ralph D. Powell, Jr., Pathologic Anatomy Branch
* Dr. Elizabeth W. Chu, Pathologic Anatomy Branch
* Dr. Philip M. Grimley, Pathologic Anatomy Branch
* Dr. Costan W. Berard, Pathologic Anatomy Branch
* Dr. Jean E. Todd, Pathologic Anatomy Branch
+ Dr. Deborah F. Carpenter, Resident, Pathologic Anatomy Branch
+ Dr. Franklin D. Curl, Resident, Pathologic Anatomy Branch
+ Dr. Harold J. DeBlanc, Resident, Pathologic Anatomy Branch
+ Dr. John M. Easton, Resident, Pathologic Anatomy Branch
+ Dr. Andrew E. Horvath, Resident, Pathologic Anatomy Branch
+ Dr. Wm. Thomas Sparrow, Resident, Pathologic Anatomy Branch
° Dr. Leon Sokoloff, Laboratory of Pathology and Histochemistry, NIAMD
° Dr. Harold R. Stanley, Jr., Clinical Director, NIDR
° Dr. Lewis E. Lipkin, Head, Section on Pathology, Perinatal Research Branch, NINDB
° Dr. William C. Roberts, Chief, Laboratory of Pathology, NHI
° Dr. William Rosenblum, Laboratory of Neuropathology, NINDB
° Dr. Marius Valsamis, Director of Laboratories, Brooklyn State Hospital, Brooklyn, New York (Consultant in Neuropathology)

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* - These Pathologists constitute the staff of the Department of Pathologic Anatomy, Clinical Center (Pathologic Anatomy Branch, NCI), and spend a major portion of their time in the service and diagnostic work of the Department.
+ - These Physicians are full time Residents in the Department of Pathologic Anatomy.

° - These Associate Pathologists spend part time in the activities of the Department of Pathologic Anatomy. In addition, several pathologists on the staff of the Laboratory of Pathology, NCI; i.e., Drs. Dawe, Banfield, O'Gara and Stanton, are available for consultation with the staff of the Department of Pathologic Anatomy.

Project Description:

The service functions of the Department during the period April 1967 through March 1968 included:

(a) 311 autopsy examinations. Included in this report is a table which shows the number of autopsies performed for each Institute. The residents perform nearly all of the postmortem dissections under supervision of the staff. A gross review is held by the staff and residents each morning at 8:30. The residents review the microscopic slides of each autopsy with one of the staff before completing the autopsy protocol.

The staff assists the residents in preparing for the numerous clinical conferences in which the Department participates.

(b) 5000 specimens were accessioned in the Surgical Pathology Section. A complete listing of these specimens, by Institute, is given in the accompanying table. These specimens are initially examined and described by a resident and their reports are checked by the staff. Associate pathologists from other laboratories or sections are frequently consulted about problems in diagnosis in surgical pathology and/or assume responsibility for handling certain tissues removed for specific research purposes.

(c) Cytopathology Service: The report for this service is Serial No. NCI 852.

(d) Electron Microscopy: Dr. Philip M. Grimley

1. Application of electron microscopy in pathologic anatomy.

(1) The efficacy of glutaraldehyde fixative in preserving tumor surgical specimens for electron microscopy has been demonstrated. Controlled studies of fixative action are in progress with Dr. Robert Chambers.

(2) Studies of human cytology materials, particularly pleural and ascitic fluids, are in progress. Preliminary results indicate that excellent preservation of these materials for ultrastructural study is routinely feasible.

(3) Ultrastructural studies of several neoplasms have been correlated with clinical and functional information (e.g. thyroid carcinoma, paraganglioma, endocrine tumors).

(4) Ultrastructural studies of kidney biopsies, microorganisms and certain sarcomas have contributed supplementary information of diagnostic import.

2. Residency Training Program

During the past year all residents have rotated through the electron microscope laboratory. Technique of tissue preparation and microscopy were demonstrated, and opportunities for practical experience offered. Interpretation of electron micrographs representing tumor surgical or cytology materials was introduced and will form the basis of future residency training. Several residents have actively participated in ultrastructural studies.

(1) Dr. Carpenter -- examination of endometrial biopsies at various stages in the menstrual cycle.

(2) Dr. DeBlanc -- study of lipid storage diseases.

(3) Dr. Bruce -- description of asteroid inclusion bodies in sarcoidosis and other conditions.

(e) Organization of histology slide files.

One of the important functions of the Pathology Department is to keep the accumulated files of histology sections in order. This is particularly true of our department in which, because of research interests, many of the histology slides are reviewed for clinical and pathological studies. Mr. James Banks is responsible for refiling each of the histologic sections in our files and for filing the gross photographs and photomicrographs in our department. It is estimated that we now have approximately three-fourths of a million slides.

(f) Special study set file.

The department has collected a number of special study sets over the years which are used by both the staff and the residents for review purposes. These study sets have now been organized so that they can be used more conveniently and, in addition, a completely coded, cross-indexed diagnostic file for each of the sections in the study set has been established. It is planned to add additional study sets of both human and animal material and thereby create a valuable asset for pathology study and review in the department. Dr. Todd is helping organize the special study set collection.

Objectives: The objectives of the Department of Pathologic Anatomy in the Clinical Center are threefold: first, to furnish a diagnostic service; second, to aid from a morphological standpoint the various clinical research problems which are under study in the Clinical Center; and third, independent research. Standard methods are used in describing, fixing and sectioning

various human organs and tissues and in addition a wide variety of special staining procedures are used.

Distribution of human organs and tissues:

At present there are approximately 66 scientists in the various laboratories at N.I.H. who have requested particular tissues from surgical and postmortem services. On many occasions it is possible to furnish these investigators with fresh human material. A list of these investigators is kept by the secretarial staff and telephone calls are made to the various investigators whenever surgical or postmortem tissues are available.

Photographic Services of the Department:

During the past four years a Zeiss Ultraphot camera microscope has been used for photomicrography. This machine has been calibrated and the procedure for its use has been explained to each of the residents and staff members of the department. It has proven to be a fine addition because it is semi-automatic and can be used by individual members of the department without the direct help of a professional photographer. During the past year the members of our staff have taken about 210 photomicrographs and in general these have been of excellent quality. These photomicrographs have materially increased the quality of case presentation at all of the conferences in which members of the department participate. Mr. Edwards develops a limited number of the color photographs and photographs taken by the staff and residents. This results in better daily and weekly conferences because key photographs are available in one or two days.

Pathology Data Retrieval:

Details about the functioning and development of the pathology data retrieval system have been given in the last two annual reports. A number of requests for data from the surgical pathology, cytology and autopsy data files were received during the past year. There was some delay in filling a number of these requests during the last year due to the fact that the Central Data Processing Division was converting from the Honeywell 800 computers to the IBM 360 computer. This necessitated converting all existing data files to the new system. The responsibility for the conversion of the pathology data files was given to Mr. Martin Epstein. He has now completed this conversion and the retrieval of pathology data is once again operational.

Recently we have started pilot runs of the system to code the pathology diagnoses by computer. Parts of the programming and development of this system represent several years of work and planning. It seems quite certain now, as a result of these pilot studies, that the development of this system will be successful and that the computer will be able to code more than 90% of the pathology diagnoses from autopsy protocols, surgical pathology, cytology and electron microscopy reports.

REPORT OF PATHOLOGIC ANATOMY DEPARTMENT APRIL 1, 1967 through MARCH 31, 1968

INSTITUTE AND TYPE OF PATIENT

Service & Type of Activity	NCI IN	NCI FU	NHI IN	NHI FU	NIAMD IN	NIAMD FU	NIAID IN	NIAID FU	NINDB IN	NINDB FU	NIMH IN	NIMH FU	NIDR IN	NIDR FU	NICHD IN	NICHD FU	REF IN	REF FU	TOTAL IN
Postmortem-Pathologic Anatomy Service																			
No. of autopsies performed*	166		115		10		4		7		0		0		1		8		311
Blocks of tissue	9863		1946		1119		224		652		0		0		138		74		14016
Slides	23194		4253		2961		512		1677		0		0		456		182		33235
Slides actually stained	13176		2172		1654		296		866		0		0		269		110		18543
Surgical Pathology																			
Accessions	3818		268		327		173		72		3		307		0		32		5000
Slides H-E staining	15491		494		1692		533		229		30		416		0		73		18958
Frozen sections	454		8		64		8		6		0		0		0		3		543
Special procedures	4234		216		569		300		79		0		12		0		21		5431

*129 Submitted autopsies: 42 NCI; 79 NHI; 1 NIAID; 7 REF

The list of specific requests together with the name of the physician or scientist initiating the requests which were processed during the past year follows. The enumeration of these requests is continued from the list attached to last year's annual report.

144. All lymphomas (surgical pathology file) NOS in lymph nodes. Drs. Berard and Thomas.

145. Cases of polycystic kidney from the autopsy file. Drs. Carpenter and Thomas.

146. All skin diagnoses from the surgical pathology file for Dr. Kierney.

147. Complete output of all cytological diagnoses together with surgical pathology and autopsy diagnoses. Drs. Malmgren and Berard.

148. Cases of parathyroid, adenoma, hyperplasia (primary) carcinoma. Dr. William Hammond.

149. Cases of colon, rectal and anal 1° cancers or 1° benign tumor from the autopsy and surgical files for Dr. William Hammond.

150. Parathyroid, all diagnoses from surgical file for Dr. William Hammond.

151. Histoplasmosis or histoplasmosis capsulatum from autopsy file for Drs. Rabson and Thomas.

152. Prostate, primary malignant tumor from autopsy file for Dr. Fraley.

153. All melanomas from both autopsy and surgical files for Dr. Alfred Ketcham.

154. Aspergillosis from autopsy file for Dr. Vogel.

155. All cases of Aspergillosis for Dr. William Thomas Sparrow from autopsy file.

156. All breast lesions from both autopsy and surgical files for Dr. Jean Todd.

157. Pancreas in diabetic hyalinization of islets from autopsy file for Dr. George Glenner.

158. Brain from Alzheimer's disease from autopsy file for Dr. George Glenner.

159. All cases of spleen.

Conferences:

The staff and residents take an active part in numerous Clinical Center and Institute conferences. In addition, there are several conferences regularly held in the department, including a Surgical Pathology Conference, Neuropathology Conference and Joint Pathologists Conference.

Publications and Other Activities:

A. Dr. Louis B. Thomas:

1. Fraumeni, J.F., Jr., and Thomas, L.B.: Malignant bladder tumors in a man and his three sons. J.A.M.A. 201: 507-509, Aug. 1967.

2. Perry, S., Thomas, L.B., Johnson, R.E., Carbone, P.P., and Haynes, H.A.: Hodgkin's disease. Ann. Intern. Med. 67: 424-442, Aug. 1967.

3. Ziegler, J.L., Carbone, P.P., Berard, C.W., and Thomas, L.B.: Burkitt tumor in the United States: Diagnosis, treatment and prognosis. E. African Med. J. (In press).

4. Hoye, R.C., Thomas, L.B., Ketcham, A.S., and Riggle, G.C.: The effects of neodymium laser on normal liver and Vx2 carcinoma transplanted into the liver of experimental animals. (In press).

5. Member, Committee on Nomenclature and Classification of Disease, sponsored by College of American Pathologists.

6. Member, Committee on Cancer Staging and End Results Reporting.

7. Chairman, Subcommittee to revise the American Cancer Society book entitled "Manual of Tumor Nomenclature and Coding".

8. Member, Pathology Review Panel for the National Lymphoma Chemotherapy Task Force (NCI).

9. Member, Biometry and Epidemiology Contract Review Committee (NCI).

Current projects which will lead to publication:

1. Clinicopathologic study of Hodgkin's disease. In collaboration with Dr. C. W. Berard, Dr. R. Johnson, Dr. P. Carbone and Dr. V. DeVita.

2. Clinicopathologic study of macroglobulinemia in collaboration with Dr. J. Fahey, Dr. E. Miller, Dr. C.W. Berard and Dr. T. Dutcher.

3. Clinicopathologic study of Burkitt's tumor in collaboration with Dr. P. Carbone and Dr. C.W. Berard.

4. Epidemiology of cancer of the cervix in the Negro. In collaboration with Dr. H.L. Stewart and Dr. L. Dunham.

5. Myelofibrosis in patients with chronic myelogenous leukemia. In collaboration with Dr. H. Gralnick and Dr. C. Vogel.

6. Final editing of 1968 Edition Manual of Tumor Nomenclature and Coding. To be published by American Cancer Society.

7. Development of a system for computer coding of pathology diagnoses. In collaboration with Dr. J.E. Todd, Dr. A. Pratt and staff at the DCRT.

B. Dr. Richard A. Malmgren:

See separate report for Cytopathology Service, Serial No. NCI 852.

C. Dr. Alan S. Rabson:

1. Strohl, W.A., Rabson, A.S., and Rouse, H.: Adenovirus tumorigenesis: Role of the viral genome in determining tumor morphology. Science 156: 1631-1633, June 1967.

2. Deftos, L.J., Rabson, A.S., Buckle, R.M., Auerbach, G.D., and Potts, J.T.: Parathyroid hormonal production in vitro by human parathyroid cells transformed by SV40. Science 159: 435-436, Jan. 1968.

3. Paulson, D.F., Rabson, A.S., and Fraley, E.E.: Viral neoplastic transformation of hamster prostate tissue in vitro. Science 159: 200-201, Jan. 1968.

4. Guggenheim, M.A., Friedman, R.A., and Rabson, A.S.: Interferon: Production by chick erythrocytes activated by cell fusion. Science 159: 542-543, Feb. 1968.

5. Malmgren, R.A., Rabson, A.S., and Carney, P.G.: Survey of human cancer patient sera for adenovirus "T" antibody by immunofluorescence. Int. J. Cancer 3: 30-34, Jan. 1968.

6. Orme, S.K., Wells, S.A., Jr., and Rabson, A.S.: In vitro neoplastic transformation of hamster pineal tissue by oncogenic DNA viruses. Cancer (In press).

7. DiPaolo, J.A., Rabson, A.S., and Malmgren, R.A.: In vitro viral transformation of chemically transformed cells. J. Nat. Cancer Inst. (In press).

D. Dr. Ralph D. Powell, Jr.:

1. Vogel, C.L., Cohen, N.H., Powell, R.D., and DeVita, V.T.: Pneumocystis carinii pneumonia. Ann. Int. Med. 68: 97-108, Jan. 1968.

2. Carpenter, D.F., Brubaker, L.H., Powell, R.D., Jr., and Valsamis, M.P.: Phycomycotic thrombosis of the basilar artery. Neurology (In press).

Current projects which will lead to publication:

1. Interstitial Emphysema: A study of physiological factors involved in experimental induction of the lesion in vivo. In collaboration with Dr. Edgar J. Caldwell (not NIH) and Dr. John P. Mullooly, NHI.

2. Induction of ovulation in infertile women by administration of gonadotrophic hormones: A correlation of clinical state with morphologic changes in endometrium and ovaries. In collaboration with Dr. John R. Marshall, Surgery Branch, NCI.

3. A comparison of surgery and combined surgery-radiotherapy in the treatment of endometrial adenocarcinoma. In collaboration with Dr. John R. Marshall, Surgery Branch, NCI.

4. Renal biopsy in lupus nephritis: Assessment of morphologic variability. In collaboration with Dr. R. Black (CC), Dr. W. Buchanan (not NIH), and H. Pettigrew, NCI.

5. Myocardial mechanics: correlation of structure with function. In collaboration with Dr. D. Patel, NHI.

E. Dr. Philip M. Grimley:

1. Grimley, P.M., and Glenner, G.G.: Histology and ultrastructure of carotid body paragangliomas. Cancer 20: 1473-1488, Sept. 1967.

2. Grimley, P.M.: Precision ultramicrotomy of narrow specimen embedments for electron microscopy -- theory and practice. J. Royal Microscopical Soc. 87: 383-391, Dec. 1967.

3. Grimley, P.M., and Glenner, G.G.: Ultrastructure of the human carotid body. A perspective on the mode of chemoreception. Circulation (In press).

4. Kohler, P.O., Grimley, P.M., and O'Malley, B.W.: Protein synthesis: Differential stimulation of cell specific proteins by estrogen and progesterone in epithelial cells of the chick oviduct. Science (In press).

5. Vietzke, W., Gelderman, A.H., Grimley, P.M., and Valsamis, M.: Toxoplasmosis complicating malignancy. Experience at the NCI. Cancer (In press).

rrent projects which will lead to publication:

1. Development of an RNA virus

   Experiments in progress indicate that development of Semliki Forest virus occurs in association with unique intracellular membrane structures. The origin and fate of these structures is being investigated by means of electron microscopic tracer and autoradiographic techniques.

2. Studies of thyroid medullary carcinoma.

   Medullary carcinoma of the thyroid is unusual in several respects. It is slow growing, produces an amyloid stroma and lacks histologic resemblance to other thyroid tumors. We are interested in its potential to synthesize amyloid as well as indications of thyrocalcitonin activity. Cells from this tumor have been established in vitro for purposes of ultrastructural, biochemical and immunologic study.

F. Dr. Costan W. Berard:

1. Roberts, W. C., Berard, C.W., and Braunwald, N.S.: Dis. of Chest 51: 439-440, Apr. 1967.

2. Brown, R.S., Haynes, H.A., Foley, H.T., Godwin, H.A., Berard, C.W., and Carbone, P.P.: Hodgkin's disease: Immunologic, clinical, and histologic features of 50 untreated patients. Ann. Intern. Med. 67: 291-302, Aug. 1967.

3. Roberts, W.C., and Berard, C.W.: Gas gangrene of the heart in clostridial septicemia. Amer. Heart J. 74: 482-488, Oct. 1967.

4. Ziegler, J.L., Carbone, P.P., Berard, C.W., and Thomas, L.B.: Burkitt tumor in the United States: Diagnosis, prognosis and treatment. E. African Med. J. (In press).

5. Member, Pathology Panel of the Lymphoma Task Force of the NCI.

6. Member, American Joint Committee for Staging and End Results Reporting of Hodgkin's disease.

Current projects which will lead to publication:

1. A detailed immunochemical and histopathologic study of three patients with multiple anomalous immunoglobulins. In collaboration with Dr. J. L. Fahey, NCI.

2. A histopathologic and cytochemical study of 20 American cases of Burkitt's lymphoma. In Collaboration with Dr. L. B. Thomas and Dr. J. M. Bennett, CP, CC.

3. A clinical, histopathologic and hematologic study of 46 cases of macroglobulinemia. In collaboration with Dr. L. B. Thomas and Dr. J. L. Fahey, NCI.

4. A histopathologic and clinical study of 12 cases of Wiskott-Aldrich syndrome. In collaboration with Dr. T. Waldmann, NCI and Dr. L. B. Thomas.

5. A histopathologic and clinical study of approximately 250 cases of Hodgkin's disease. In collaboration with Dr. L. B. Thomas.

6. A review of the correlation between exfoliative cytology and histopathology in patients evaluated at the NCI over the past 15 years. In collaboration with Dr. R. A. Malmgren and Dr. E. W. Chu.

7. Correlation of cytologic changes with cultural results in specimens screened for viruses.

8. Cytologic changes secondary to therapy with Myleran and/or Dibromannitol.

G. Dr. Jean E. Todd:

Current projects which will lead to publication:

Electron microscopy of surgical specimens from the Clinical Center with special emphasis on malignant tumors of known and unknown histogenesis, liver and bone marrow biopsies.

H. Dr. Deborah F. Carpenter:

1. Carpenter, D.F., Golden, A., and Roberts, W.C.: Quadrivalvular rheumatoid heart disease associated with left bundle branch block. Amer. J. Med. 44: 922-929, Dec. 1967.

2. Carpenter, D.F., Brubaker, L.H., Powell, R.D., Jr., and Valsamis, M.P.: Phycomycotic thrombosis of the basilar artery. Neurology (In press).

Current projects which will lead to publication:

1. Study on cystic fibrosis with Dr. J.H. Edgcomb (formerly of NIH), and Dr. S. Handwerger (NIAMD).

2. Case report of a case of malignant histiocytosis with Dr. W. Levis (Dermatology Branch) and Dr. R. Kagen (Radiation, NCI).

3. Electron microscope study of endometrium in various stages of cycle and after stimulation with drugs and with chorionic gonadotropin. In collaboration with Dr. P.M. Grimley and Dr. J.R. Marshall (Surgery Branch).

4. Case report and study of effect of human chorionic gonadotropin on fetal testis. In collaboration with Dr. R.D. Powell, Jr. and Dr. J. Wider (Endocrinology, NCI).

5. Study of NZB mice in collaboration with Dr. Norman Talal (NIAMD) and Dr. Parker Staples (NIAMD).

I. Dr. Franklin D. Curl:

1. Pollay, M., and Curl, F.: Secretion of cerebrospinal fluid by the ventricular ependyma of the rabbit. Amer. J. Physiol. 213: 1031-1038, Oct. 1967.

2. Curl, F., and Pollay, M.: Transport of water and electrolyte between brain and ventricular fluid in the rabbit. J. Exp. Neurol. (In press).

Current projects which will lead to publication:

1. Case review on cerebral toxoplasmosis with Dr. Arthur Levine, NCI.

J. Dr. Harold J. DeBlanc:

1. Study on a strain of CBA mice which produce a genetically transmitted storage-like disease.

2. Two or three human cases.

(a) An angiofibroma which presented clinically as a carotid body tumor and will be an unusual case of regional ileitis.

K. Dr. John M. Easton:

1. Davis, W.W., Newsome, H.H., Jr., Wright, L.D., Jr., Hammond, W.G., Easton, J.M., and Bartter, F.C.: Bilateral adrenal hyperplasia as a cause of primary aldosteronism with hypertension, hypokalemia and suppressed renin activity. Amer. J. Med. 42: 642-647, April 1967.

2. Fraumeni, J.F., Jr., Vogel, C.L., and Easton, J.M.: Sarcomas and multiple polyposis in a kindred. Arch. Intern. Med. 121: 57-61, Jan. 1968.

Current projects which will lead to publication:

1. Case report dealing with "heavy chain disease", a condition in which there is abnormal production of a fragment of the "heavy" polypeptide component of 7S gamma globulin. In collaboration with Dr. Daniel Ein, NCI.

L. Dr. Andrew E. Horvath:

Current projects which will lead to publication:

1. Review of 80 cases of choriocarcinoma in regard to cardiac metastasis. In collaboration with Dr. W. C. Roberts (NHI).

2. Moloney sarcoma virus purification and characterization with Dr. R.M. Friedman, NCI.

M. Dr. Wm. Thomas Sparrow:

Current projects which will lead to publication:

1. Working on a case report with Dr. W. Rosenblum (NINDB) and

Dr. D. Pleasure (NINDB) on a patient with subacute necrotizing encephalomyelopathy.

2. Review of 90 or so autopsies of patients with Aspergillosis. In collaboration with Dr. C. Vogel (NCI) and Dr. R. Johnson (NCI).

N. Dr. Daniel W. Bruce:

1. Bruce, D.W., and Edgcomb, J.H.: Pancytopenia and generalized sepsis following treatment of cancer of the urinary bladder with instillations of triethylene thiophosphormide. J. Urol. 97: 482-485, March 1967.

O. Mary C. Bowling, B.S., M.T., (ASCP):

1. Bowling, M.D.: Histopathology Laboratory Procedures for the Pathologic Anatomy Branch of the National Cancer Institute. U.S. Department of Health, Education, and Welfare, Public Health Service Publication No. 1595. Washington, D.C., Government Printing Office, 1967, 125 pp.

2. Chambers, R.W., Bowling, M.D., and Grimley, P.M.: Glutaraldehyde fixation in routine histopathology. Arch. Path. 85: 18-30, Jan. 1968.

Serial No. NCI 857
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Interstitial Pulmonary Emphysema: Acute and chronic morphological and physiological changes induced in vivo in rabbits by regulated elevation of intratracheal air pressure

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Edgar J. Caldwell, M.D. and John P. Mallooly, Ph.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 1/4
Professional: 1/8
Other: 1/8

Project Description:

Objectives:

1. To determine under standard conditions the amount of airway pressure necessary to produce dissection of air into the pulmonary interstitial space.

2. To characterize the lesion morphologically with regard to location, extent, shape, etc.

3. To study the concomitant physiologic changes and determine the pathogenetic significance of such variables as: pulmonary artery pressure, pleural space pressure, lung volume and absolute airway pressure.

4. To expose animals on a chronic basis to elevated airway pressures to determine if such stress will induce emphysema of the type commonly seen in adult humans and to study the evolution of the interstitial emphysema.

Major Findings:

1. The incidence of interstitial emphysema is directly related to the applied airway pressure and is high above 50 cm. of water pressure.

2. The incidence of interstitial emphysema increases directly with the degree of expansion (volume) of the lung.

3. The incidence of interstitial emphysema does not seem to be related to the pulmonary artery pressure.

4. Interstitial emphysema appears to heal spontaneously in spite of repeated exposure to the applied stress. Chronically stressed animals become resistant to development of interstitial emphysema.

Significance:

1. In the rabbit, airway pressures lower than those normally generated by coughing in the human or than those often created during therapeutic intermittent positive pressure breathing induce interstitial pulmonary emphysema accompanied by significant tissue destruction.

2. The observation that the incidence of interstitial emphysema increases with the degree of expansion of the lung, supports the hypothesis that pressure in the interstitial space falls as the degree of expansion of the lung increases.

3. These lesions may heal spontaneously. It may be possible to produce a state of resistance to development of the lesion through chronic exposure to elevated airway pressures.

Proposed Course:

1. Begin in vivo experiments to define more precisely the relationship of lung volume and pulmonary artery pressure to incidence of interstitial emphysema.

2. Develop techniques for measuring pressure in the interstitial space to permit monitoring of this pressure during changes in lung volume, etc.

3. Determine how much and what types of functional disturbances of breathing are produced by interstitial emphysema.

4. Determine what morphologic or physiologic changes account for the animal's acquired resistance to development of interstitial emphysema.

Serial No. NCI 858
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Induction of ovulation by gonadotropic hormones: A clinico-pathologic study

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: John R. Marshall, M.D., Charles B. Hammond, M.D., and Arnold Jacobson, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 1
Professional: 1/2
Other: 1/2

Project Description:

Objectives:

1. To study and treat women who are infertile because of failure to ovulate.

2. To induce ovulation permitting successful reproductive function through the careful use of human gonadotropins (human menopausal gonadotropin and human chorionic gonadotropin).

3. To attempt to elucidate the action of these gonadotropins upon ovulation and endocrine function of the ovary.

4. To determine the prognostic and diagnostic value of endometrial and ovarian biopsies as well as several other laboratory and clinical indicators of ovarian function.

Major Findings:

1. Ovulation appears to have been successfully induced in several previously anovulatory or oligo-ovulatory women. Pregnancy has occurred in

13 patients during gonadotropic therapy.

2. With regard to indicators of ovulation, there is considerable agreement between endometrial biopsy, urinary pregnandiol excretion, basal body temperatures, physical properties of cervical mucus and clinical signs and symptoms.

Significance:

Information is being acquired which should be of use not only in the successful accomplishment of pregnancies, but also in the prevention of undesired pregnancies.

Proposed Course:

1. To study additional patients through additional menstrual cycles to determine more accurately the optimal dosage schedule for the individual patient.

2. To study endometrial biopsies obtained at more frequent intervals throughout the menstrual cycles to evaluate similarities to normal menstrual cycles.

3. To study ovarian morphologic changes in greater detail and correlate these with gonadotropin responsiveness.

Serial No. NCI 859
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Renal biopsy in lupus nephritis: A statistical analysis of variability of disease at different sites within single kidneys

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Roger L. Black, M.D., Watson Buchanan, M.D., William M. O'Brien, M.D., and Hugh M. Pettigrew

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 1/12
Professional: 1/24
Other: 1/24

Project Description:

Objectives:

1. To assess the non-uniformity of distribution of morphologic changes throughout the kidney affected by lupus nephritis.

2. To determine the effect of this non-uniform distribution upon morphologic variability among renal biopsies obtained from different sites in a single kidney.

3. To determine the limitations imposed by this variability upon the diagnostic significance of differences noted between two biopsies taken from the same kidney at different times, for example, before and after therapy.

Major Findings:

Twenty-nine morphologic features for each of 96 renal biopsies from 16 kidneys have been recorded by each of three observers. The statistical analysis of these data is in progress.

Proposed Course: Complete the statistical analysis of the data.

Serial No. NCI 860
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Myocardial mechanics: Relationships between spatial orientation of muscle fibers and dimensional changes during systole in dog left ventricle

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: Dali Patel, M.D. and Daniel D. Streeter, Jr.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 1/2
Professional: 1/6
Other: 1/3

Project Description:

Objectives:

1. To study normal function of the left ventricle of the dog heart during systole and to correlate certain functional properties of the ventricle with certain structural features of the ventricular wall. Specifically, to correlate systolic changes in: circumferential and vertical dimensions, tension developed by ventricular wall and pressure developed in ventricular lumen with: spatial orientation of the muscle fibers in the muscle layers of the ventricle.

Major Findings:

1. In the mid-transection plane of the left ventricle the wall consists of three layers: a moderately thick innermost layer of vertically oriented fibers, a thick middle layer of circumferentially oriented fibers and a thin inconstant outermost layer of vertically oriented fiber.

Significance:

The muscle fibers of the left ventricle of the dog may be easily separable into two groups, one group which tends to decrease the radius of

the ventricle and one group which tends to decrease the length of the ventricle along its verticle axis.

Proposed Course:

1. Complete plotting of the orientation of muscle fibers.

2. Determine whether it is more valid to divide fibers into three groups or to view them as a continuum of changing orientation angle.

3. Calculate force vectors for the layers of the wall and for the wall as a whole.

4. Correlate these force vectors with observed changes in ventricular dimensions and developed tensions.

Serial No. NCI 861
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Morphologic, histochemical and biological studies of neuroendocrine tumors

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: George G. Glenner, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 3/4
Professional: 1/2
Other: 1/4

Project Description:

Objectives:

1. To study the histology and fine structure of paragangliomas and pheochromocytomas.

2. To correlate the morphologic appearance with clinical, histochemical and biochemical evidence of hormone production.

3. To develop appropriate experimental systems for the study of neurohormone synthesis in neoplastic tissues.

Major Findings: Combined ultrastructural, histochemical and fluorimetric evidence suggests that tumors derived from the carotid body and other paraganglia produce catecholamines. Common ancestry of paraganglia and chromaffin ganglia is suggested by electron microscopic comparisons. Findings in the tumors provide a better basis for predicting the mechanism of chemosensation in normal paraganglia.

Significance: Tumors of the neuroendocrine ganglia are of unusual biological interest, since the synthesis of a specific product and presence of unique cytoplasmic enzymes provide a persistent marker after neoplastic

transformation. The role of stroma in tumors of the carotid body may be of considerable interest. This gland possesses a peculiar normal architecture in which primary lobules of chief cells are segregated by surrounding sustentacular elements. An experimental system might permit investigation of tumor evolution from individual nests and indicate if this is a form of field induction or single cell overgrowth. The lack of chromaffinity in certain tumors known to secrete catecholamines in large quantity is a perplexing biologic problem. Studies of tumor cells or cell cultures of neurosecretory glands may elucidate the basic biological mechanism of biogenic amine storage within cytoplasmic secretory granules.

Proposed Course:

1. To attempt development of experimental paragangliomas in dogs or other animals known to develop such tumors spontaneously.

2. To attempt tissue culture of adrenal medullary cells and determine the persistence of catecholamine production in vitro.

3. To develop a biological model suitable for the study of neurohormone sequestration and release from granule matrix binding sites.

Publications:

1. Grimley, P.M., and Glenner, G.G.: Histology and ultrastructure of carotid body paragangliomas: Comparison with the normal gland. Cancer 20: 1473-1488, Sept. 1967.

2. Grimley, P.M., and Glenner, G.G.: Ultrastructure of the human carotid body. A perspective on the mode of chemoreception. Circulation (in press).

Serial No. NCI 862
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Morphological, histochemical, and biological studies of a hormonally responsive epithelium

Previous Serial Number: Same

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Peter O. Kohler, M.D., Bert O'Malley, M.D., and J. Sri Ram, M.D.

Cooperating Units: Clinical Endocrinology Branch, NCI
Laboratory of Experimental Pathology, NIAMD

Man Years (July 1, 1967 through June 30, 1968)

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Objectives:

1. To document in detail the structural features of a hormonally responsive epithelium by light and electron microscopy and correlate alterations in morphology with the type of stimulation and degree of functional response. In particular this system utilizes immature chick oviduct stimulated in vivo or in vitro by estrogen or progesterone.

2. To determine the specific cell types responsible for secretion of biochemically assayed products (albumin, avidin, lysozyme) by means of histochemical, fluorescent antibody or radioautographic correlations.

3. To trace subcellular events during a phase of rapid multiplication and growth induced by hormone.

Major Findings:

1. An immature, ultrastructurally homogeneous epithelium (chick oviduct mucosa) gives rise to three morphologically distinctive cell types after stimulation with diethylstilbestrol (DES).

2. Formation of the protein albumin occurs in tubular gland cells and is directly related to the duration of estrogenic stimulation.

3. A specialized surface layer of goblet cells which develops under the influence of estrogen, fails to produce albumin, but is capable of producing another protein avidin when induced by progesterone.

4. A granule with distinctive ultrastructural features develops in the goblet cells after prolonged estrogen treatment. Histochemical and structural characteristics suggest an identity to ovimucin.

Significance: Control of genic expression by hormonal inducers or repressors is of profound biological significance both in the understanding of normal developmental processes and the process of neoplasia. Events in the chick oviduct are thus far unique in their susceptibility to exogenous manipulation. The system described therefore offers an unusual opportunity to observe chronologically, the progress of morphologic and functional changes during rapid growth and differentiation.

Proposed Course: More complete documentation of morphologic changes in times sequential in vivo studies will be attempted. Studies of tissue enzyme alterations are in progress. Fluorescent labeled antisera to various egg white proteins will be prepared and identification of the histologic sites of egg white protein production as influenced by the hormonal stimuli.

Publications:

1. Kohler, P.O., Grimley, P.M., and O'Malley, B.W.: Protein synthesis: Differential stimulation of cell specific proteins by estrogen and progesterone in epithelial cells of the chick oviduct. Science (in press).

Serial No. NCI 863a
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ultrastructural studies of human tumor cells

Previous Serial Number: 863

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Alan S. Rabson, M.D., and Richard A. Malmgren, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Objectives:

1. To examine the ultrastructure of tumor cells in surgical specimens or samples of body fluids.

2. To compare the morphologic features of tumor cells grown in vitro with samples of the original materials.

3. To identify the presence, if any, of particles resembling viral etiologic agents described in a variety of murine neoplasms including mammary tumors and lymphomas.

Major Findings: Methods for routine preservation of surgical tumor specimens for ultrastructural examination have been improved and methods for the concentration and electron microscopic examination of cells in body fluids have been developed.

Significance: The significance of such investigations lies in the stated objectives: Results contribute basic information regarding tumor ultrastructure and etiology.

Proposed Course: Studies of several tumor cell lines are in progress, including medullary carcinoma of the thyroid, Ewing's sarcoma and malignant lymphoma.

Publications:

1. Chambers, R.W., Bowling, M.C., and Grimley, P.M.: Glutaraldehyde fixation in routine histopathology. Arch. Path. 85: 18-30, Jan. 1968.

Serial No. NCI 863b
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Ultrastructural and biologic studies of virus infected cells

Previous Serial Number: 856

Principal Investigator: Philip M. Grimley, M.D.

Other Investigators: Alan S. Rabson, M.D., Victor Bedoya, M.D., and Albert H. Gelderman, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total: 1
Professional: 3/4
Other: 1/4

Project Description:

Objectives:

1. To examine in detail the light and electron microscopic features of cell lines displaying "herpes-like" intranuclear particles; specifically, cell lines derived from patients with malignant lymphoma.

2. To study the growth cycle of herpes simplex (HSV) infection in lymphoma cell lines.

3. To characterize the ultrastructural and cytofunctional alterations in cells infected by herpes-type viruses including human strains of cytomegalovirus (CMV) which infect patients with leukemia and lymphomas.

4. To study conditions which alter host cell susceptibility to virus infections.

Major Findings:

1. The morphologic effects of herpes simplex virus infection in lymphoma cells are quite similar to changes apparently induced by the spontaneously occurring "herpes-like" or EB virus particles. These changes

include disfigurement of the nuclear envelope and a general disturbance of cytomembrane organization and formation of type A intranuclear inclusions.

2. Infection of cells with CMV results in a delayed cytotoxic effect. Such cells continue to support the growth of the obligate intracellular parasite Toxoplasma gondii.

Significance: Comparison of cytologic reactions to infection by herpes-type viruses may elucidate the significance of EB agents detected in human lymphoid cell lines cultured in vitro. In addition the studies outlined contribute basic biologic information in the area of virus-host cell relationships.

Proposed Course:

1. To continue ultrastructural comparisons of herpes group virus development in lymphoid and other cell lines.

2. To study the growth of CMV under various experimental conditions.

Serial No. NCI 864
1. Pathologic Anatomy Branch
2. Surgical Pathology and Postmortem Service
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A comparison of surgery and combined surgery-radiotherapy in the treatment of endometrial adenocarcinoma

Previous Serial Number: Same

Principal Investigator: Ralph D. Powell, Jr., M.D.

Other Investigators: John R. Marshall, M.D. and Ralph Johnson, M.D.

Cooperating Units: Surgery, NCI
Serial No. 3802

Man Years (July 1, 1967 through June 30, 1968)

Total: 0
Professional:
Other:

Project Description:

Objectives:

1. To evaluate the effectiveness of surgery and combined surgery-radiotherapy on patients with localized endometrial adenocarcinoma.

The development of this project has been going on for two or three years but it has not actually started as yet. Since July Dr. Powell has been Chairman of the Pathology Review Committee.

Proposed Course:

Obtain approval of project by Office of the Surgeon General.

Annual Report of the Laboratory of Physiology,

GL&C, National Cancer Institute

July 1, 1967, through June 30, 1968

During this fiscal year the Laboratory has continued with studies reported previously. In addition studies on control mechanisms in protein and nucleic acid have been expanded. With new techniques available for studying cells at the subcellular level, advances have been made on the important problem of determining how normal cells function and to study mechanisms involved in these functions. At the same time the Laboratory has not lost sight of its ultimate goal, namely, the application of those experiments to neoplastic growth. The Laboratory still continues to encourage studies on a variety of systems whether they be the whole mammalian organism or whether they be microorganisms. Previous reports have indicated that what can be learned with microorganisms can be applied to studies on mammalian tissue, including human.

Cancer Physiology Section

Dr. Wollman continues his studies on the growth and function of normal and neoplastic tissue including the relation between structure and function. The principal tissue which he is studying at the present time is the thyroid gland. This tissue has been specifically chosen because it has many morphologic and biochemical markers; its growth and function can be controlled by hormones and by a variety of inhibitors; it is relatively easy to make a wide variety of functional and non-functional tumors of this tissue. At the present time he is investigating the nature of growth of the thyroid gland using morphologic and autoradiographic (tritiated thymidine) approaches; changes in the subcellular organella complement during growth and function in individual cells; relation between structure and functions in thyroid tumors; factors controlling patterns of necrosis in tumors. During the past year emphasis has been placed on changes occurring in the thyroid gland during hyperplasia and during involution of the hyperplastic gland. He has studied DNA synthesis, weight changes, morphologic changes at both light and electron microscope levels. It seems clear now that gland weight during involution does not come back to control levels for very long periods and neither does DNA content. Evidence has also been obtained for marked changes in the viscosity of thyroid colloid in some follicles during a time when the gland is in a steady state.

Dr. Rabinovitz continues his studies on control mechanisms involved in the protein biosynthetic apparatus. He has been introducing specific

biochemical lesions within the pathway of the well-characterized protein hemoglobin. The lesions thus introduced can be related to the alterations in the rate of initiation, progression at specific loci and completion of peptide chains. He has found that all these changes are reversible upon the addition of the appropriate metabolite. Globin synthesis can only be initiated if there is an adequate supply of hemin. In addition some uncharacterized membrane factor is needed. If globin synthesis cannot be initiated then polyribosome disaggregation occurs. Similarly, the deficiency or inhibition of amino acids located only near the amino-terminal end of the peptide chain will cause disaggregation. However, if the biochemical lesion is introduced only at the terminal stages of globin chain formation, an enlarged polyribosome is formed. This enlarged ribosome is thought to be due to the accumulation of ribosome prior to the lesion which results in a stretching of the messenger RNA.

Dr. Reid continues his studies on urinary excretion patterns in leukemia: The objectives are to develop methodology which will greatly broaden the chemical information obtained from urinary samples and lead to an evaluation of such information for usefulness in the clinic and the laboratories. Significant abnormalities in excretion patterns are being sought of all urines which have detectable ultraviolet absorbing components from leukemic subjects and normal subjects. The main interest is restricted to nucleic acid congeners. Analytical systems are being constructed which are powerful enough to deal with the complexity of urinary components by the application of a computational technique for multicomponent mixture analysis to urinary fractions obtained after preliminary chromatographic separation. Techniques have been developed by Dr. Reid in this program which permit quantitative estimation of the components without the necessity for chemical identification. The feature of this approach is the ability to recognize pattern abnormalities should they occur. Hence, this project hopes to employ chemical and computer technology, to resolve in a relatively simple manner the complex multicomponent nucleic acid congeners which are present in leukemic urines.

Physical Biology Section

Dr. Shack has spent the past year on two major problems. He has been studying the physical and chemical properties of a DNA-ase inhibitor prepared from human white cells. He was particularly interested in determining whether this inhibitor in the blood was present in the urines of leukemics. No significant amounts of the inhibitor was found in these urines. He has also been investigating the nature of a factor found in human serum which apparently stabilizes the DNA-ase inhibitor. Employing zonal electrophoresis and sedimentation as a means of purification, this stabilizing factor has been shown to be an alpha globulin. More recently he has been studying the nature of newly formed nucleoprotein and the manner in which it changes during the growth cycle of the cell. He has found that the portion of the nucleoprotein which contains newly formed DNA can be distinguished from and partially separated from the bulk of the nucleoprotein. This affords an opportunity to study a variety of newly formed nucleoproteins under variable conditions to determine the specificity of these new formed nucleoproteins.

Dr. Breitman has extended his studies to determine the mechanisms which control purine and pyrimidine ribo- and deoxyribonucleotide biosynthesis with particular emphasis on the role of thymine deoxyribonucleotides. This latter emphasis has been made possible by the fact that he has several strains of growing Escherichia coli, some of which are characterized by their dependence on thymine for their growth. This past year he has concentrated his efforts in four main projects: (1) what controls deoxyribonucleotide biosynthesis in growing E. coli, (2) studies on the metabolism of thymineless mutants of E. coli, (3) studies on pyrimidine metabolism in human leukocytes, (4) studies on the synthesis of phage specific enzymes. Answers to these problems should help elucidate control mechanisms of DNA replication and as a result contribute to the general study of growth and differentiation. He has shown that the effect of an increase in the intracellular deoxythymidylate pool as growing E. coli on the intracellular pools of the other deoxyribonucleotides is that the biosynthesis de novo of the pyrimidine deoxyribonucleotides (deoxycytidylate, uridylate and thymidylate) was inhibited, whereas, there were no significant changes in the corresponding purine deoxyribonucleotides. This selector inhibition suggested that ribonucleotide reductase, the enzyme catalyzing the synthesis de novo of all deoxyribonucleotides, was allosterically inhibited by deoxythymidylate. The level of this enzyme in vitro was found to be the same in cells with elevated intracellular deoxythymidylate pools compared to normal cells. This is, therefore, interpreted to mean that the mechanism of enzyme repression was eliminated as the control mechanism and the action of deoxythymidylate as an allosteric effector is the most plausible to explain the selective inhibition by deoxythymidylate of pyrimidine deoxyribonucleotide biosynthesis in the intact cell.

Dr. Peterkofsky, who joined the Laboratory as a Research Fellow in July 1967, and who will probably join the staff soon, has brought a new approach to the Laboratory. She has been interested in studying factors regulating the initiation of specific protein synthesis during embryonic development. Her chief objectives are to seek as yet unidentified factors which are responsible for initiating and controlling the synthesis of tryptophan pyrrolase, an enzyme of the liver and the structural protein in embryonic tissues, namely collagen. She is at present developing techniques or methods whereby one can detect small amounts of tryptophan pyrrolase and collagen. She has devised a new assay for tryptophan pyrrolase which is based on the release of radioactively labelled formic acid from 2-C^{14}-L-tryptophan treated sequentially with the enzymes tryptophan pyrrolase and formyl-L-kynurenine formylase. Chick embryos at various stages of development are being tested for the presence of enzyme tryptophan pyrrolase. For the studies on collagen, collagenase, an enzyme which specifically hydrolyzes certain peptide bonds in collagen will be employed. These studies should advance our knowledge on some of the regulatory mechanisms which are functioning in normal rapidly proliferating embryonic cells which result in differentiated cells carrying out specific functions. In addition the possible role of glucocorticoid hormones in the developmental regulation of tryptophan pyrrolase will be investigated, since these hormones are known to increase the rate of synthesis of this enzyme in the liver.

Radiation Biology Section

Studies by Dr. Elkind on the responses of Chinese hamster cells to irradiation has continued and the effect of two bifunctional alkylating agents completed. He has previously shown that sublethal damage accumulation is a necessary requirement for cell destruction by X-rays; that sublethal damage is repairable and is demonstrable by a fractionation technique. Sulfur mustard, $S(C_2H_4Cl)_2$, an alkylating agent, will also cause cell killing and also involves damage accumulation and similarly sublethal damage is repaired. On the other hand, very little damage accumulation is required for cell killing with nitrogen mustard, $CH_3N(C_2H_4Cl)_2$, and consistent with this sublethal damage, repair is small when dose fractionation is employed. These data lead to the following generality: if sublethal damage must be accumulated to kill a mammalian cell, this damage is repairable. X-rays and nitrogen mustard, two agents which do not interact, involve quite different capacities for and abilities to repair sublethal damage; and two agents which do interact, e.g., X-rays and actinomycin D, appear to have similar abilities to repair sublethal damage. Although actinomycin D affects lethal and sublethal damage, RNA synthesis is not completely arrested. Puromycin which has a larger effect on RNA and DNA synthesis, does not suppress sublethal damage repair. Thus, it appears that actinomycin D is effective because of its ability to bind DNA rather than its interference with metabolic processes.

Dr. Withers has been engaged in studies on the response of mouse intestinal mucosa to irradiation. He has found that the radiosensitivity of this tissue is similar to that of other normal and malignant mammalian cells except that the intestinal cells have a greater ability to repair sublethal injury. By dividing a dose of X-rays into multiple fractions, a disproportionate benefit will accrue to the intestinal cells as compared with some malignant murine cells so far studied in vitro. Therefore, the importance of optimizing the size of the individual fractions of a dose-schedule in radiotherapy is indicated. It also predicts that techniques which reduce the capacity of cells for sublethal injury repair will cause a disproportionately adverse effect on the intestine.

Dr. Smith's researches have been concentrated on two broad problems (a) determining the kinetics of damage and recovery on the hematopoietic system following total body irradiation and (b) effects of specific chemical agents on the response of tumor bearing mice to irradiation. The importance in cancer therapy of the ability of the hematopoietic stem cell population to recover from radiation or chemotherapeutic agents is apparent. Part of these studies are devoted to study the responses to those agents and the processes involved in recovery. Applying the ability of stem cells to form colonies in the spleen, effects of various agents used in chemo-therapy and the response on tumor bearing mice to irradiation can be made and analysis of possible interaction determined. Employing the vinca alkaloids, vinblastine and vincristine, she has shown that on administration of the latter, two days prior to irradiation the colony forming units began to increase immediately without the usual post-irradiation lag phase. The colony-forming unit stem cell assay depends upon the transplantability of

the cells. Vinblastine definitely reduces this transplantability. These two alkaloids, individually proved to increase the effectiveness of irradiation of mice bearing the lymphosarcoma P1798. Tumor-bearing mice injected with vinblastine two days before irradiation were shown to be more tolerant to a high irradiation dose.

Dr. Riesz continues his studies on locating the biological active centers of proteins and DNA by exposing them to high doses of ionizing radiation. This procedure permits the molecular damage in these molecules and the effect on active sites to be determined. This work has been performed in the dry state where the energy is absorbed directly by the macromolecule and where diffusion is limited. However, in dilute aqueous solution the effects of ionizing radiation on these biologically important molecules are due entirely to the reaction of freely diffusible radicals formed by the radiolysis of water. Since the damage done by ionizing radiation to the macromolecule in the cell is probably due to both of these processes, it becomes necessary to study the damage produced in the dry state and those produced by the reaction of water radicals as determined in aqueous solution. During the past year he has studied the effect of temperature and conformation on the free radical distribution in the dry state of several gamma irradiated dry state. At 25° C each native protein has a characteristic tritium distribution (a means of locating radiation damage). At -79° C the free radical distribution of native proteins are equivalent to those of denatured proteins at 25° C. These results can best be explained by considering the effect of conformation on the intra- and intermolecular radical migration which occurs when the protein is warmed from -79° C to 25° C. Tritiated ribonuclease and lysozyme showed a surprising degree of homogeneity with most of the radioactivity coinciding with the major protein components. As a problem of possible interest, this technique may prove useful in the preparation of tritiated proteins which could be employed in a wide variety of biochemical investigations.

Studies of immunogenetic factors affecting transplantation of hematopoietic tissues in irradiated mice and the modification of functional capacity of bone marrow cells are being continued by Miss Uphoff. The objective of this study is to determine the factors which alter the functional capacity of immunologically competent bone marrow cells with the ultimate goal of increasing the feasibility of transplanting bone marrow and other organ tissues. She has recently been studying various aspects of the allograft reaction with the aim of developing new methods of altering the immune response to study possible methods of modifying tissue antigenicity and to study effect of mixing allogeneic cells. Transplantation of hematopoietic tissues into irradiated mice was used to test donor and host responses. Selected inbred strains or hybrid mice were used to demonstrate mechanisms, sensitivity and specificity of tissue reaction. High leukemic strains were used to demonstrate the effects of various treatments on the incidence of neoplasms. Marrow cells were preincubated in vitro with cellular antigens for various intervals of time and temperatures prior to inoculation into lethally-irradiated recipients and following the survival pattern followed. Survivors were tested with skin hemografts to demonstrate tolerance or chimerism (i.e., a successful permanent marrow allograft). Incubation of marrow cells

with washed allogeneic erythrocytes markedly affected the protective capacity of the inoculated bone marrow. Tolerance was demonstrated by skin allografts while the "sensitization" of cells was demonstrated by an accelerated graft-versus-host reaction.

With this report Dr. Maxwell will conclude his studies on the effects of ionizing radiation on the cell and on systems in vitro capable of stimulating cellular functions. He has been mainly concerned with the mammalian ribosome of rat liver. He has been examining the physical changes produced in the ribosome and the performance of the ribosome in a cell free protein synthesizing system. The ribosomes were isolated from rat liver after high doses of total body irradiation. He has found that the irradiated ribosome apparently retains its ability to recognize the genetic code, i.e., the C^{14} amino-acid incorporations of seven amino acids other than phenylalanine were not stimulated by poly-U; that the polysomal fragments produced by radiation from larger polysomes still react in the incorporation system, presumably to produce protein.

Dr. Draper's studies on antibody formation following multiple irradiation and immunization procedures have continued. During the past year, two immunological problems have been studied; (1) the comparison of the depressive action of acute and fractionated irradiation on mammalian immune processes, particularly on the formation of natural Forssman hemolysin in rabbits. This includes studies on the modification of the recovery of immune competence after irradiation by added immunosuppressive drug treatment and organ extirpation procedures. These studies have been extended to quantitate the irradiation effects at the cellular level and to obtain data on the tissue source of natural hemolysin and (2) the qualitative changes in antibody molecules subjected to high energy electron irradiation. Specifically, these studies will provide information on the factors determining the binding affinity or avidity of hemolysin for the target red cells.

Energy Metabolism Section

The all important studies by Drs. Morrison and Millar on the impact of the growing tumor on the various parameters in the normal host continue. They have previously shown that the tumor bearing rat shows a declining food intake, a sustained water intake, and a drastic depletion of host tissue. They are undertaking a study on the physiology of food and water intake regulation. They have recently shown that with feeding as an activity, the total spontaneous activity of rat progressively diminishes with growth of a tumor; that the reduction of food intake closely follows the diminution of activity. The amount of reduction of activity is closely related to the estimated rate of production of lactic acid by the tumor. This may infer a host debilitation as a direct result of an identifiable load imposed on it by the tumor. The tumor-bearer sustains or even increases its water intake while its food intake is declining. It was shown that this behavior of water intake is a reproducible feature of rats subjected to restriction or deprivation of food. Much of the high water intake of tumor-bearers can therefore be attributed to the decline of food intake

if that is considered as a physiological restriction (restricted by the metabolic load of the tumor) rather than as a low spontaneous intake. There remains, however, a fraction of the high water intake that cannot be accounted for in this way and appears to be a more specific consequence of the tumor.

Office of the Chief

When Dr. A. W. Pratt left the Laboratory to become the Director of the Division of Computer Research and Technology, Dr. F. K. Millar was placed in charge of the Data Processing which the Laboratory made available to members of the NCI. The general problems which she handled may be arbitrarily divided into three categories: (1) data processing of raw data, (2) storage and retrieval of large amounts of data, and (3) testing of mathematical models derived from experimental results. Some examples of direct processing of raw data, which are currently in use by the staff are: (1) The processing of punched paper tape output from liquid scintillation spectrometers: Existing programs convert data to disintegrations per minute, sum groups of samples as directed by the investigator, and calculate final results. There are programs to handle data from double isotope labelling experiments. (2) Detailed, standard, statistical analyses of data from various experimental sources (immunology, radiology, enzyme kinetics): Programs differ according to the kinds of data and the needs of the investigator. (3) Processing of punched paper tape output from the analog-digital converter of a small animal calorimeter: Output is checked for machine errors; O_2 readings are converted to a format compatible with an X-Y Recorder for automatic plotting, so that the digital read-outs can be directly compared with the analog O_2 trace from the calorimeter. Work is in progress to convert the instantaneous O_2 readings into a "square wave" form. A computer application in the category of information storage and retrieval concerns the body of data from the chorio-carcinoma chemotherapy study. Approximately one-third of this data (medical histories and clinical records) has been processed and stored on disk-pack. No further work on this data has been done during the past year. In the category of mathematical model building, attempts are being made to estimate the metabolizing mass of a solid tumor. Using the computer, data derived from daily tumor measurements are fitted to a mathematical function (e.g., the Gompertz equation) and the results predicted by theoretical solutions are tested. Another example of this kind of computer application is that of the Varigrad, the device for producing a variable gradient for chromatographic elution. Both least squares fit and linear programming have been tried as means of predicting the initial eluent concentrations required by the system in order to yield a gradient curve of desired shape.

Dr. White has continued his studies on tumor-host relationship employing the Walker 256 tumor. He has now shown that rats bearing the Walker 256 tumor and fed a diet in which the source of protein was defatted lyophilized Walker tumor, grew larger tumors and gained more body weight (minus tumor) than did similar tumor-bearing animals injesting a diet containing casein or casein supplemented with 0.35 or .70% sodium chloride. Average daily water intakes of animals in the tumor diet was 40 per cent higher than the other group. Animals on the casein or casein-salt diet all showed

remarkably equivalent water intakes. Higher concentrations of NaCl (.70%) resulted in smaller tumors and lowered body weight gains.

The absolute dependence of the smooth operation of the research program on the electronic, machine and glass blowing shop was again evident this year. Design and fabrication of gear unavailable on the market, the modification of standard gear, emergency repairs all contributed to the success that the Laboratory has enjoyed this fiscal year. Among some of the important items fabricated are the following:

Design and fabrication of a 2-stage water still with automatic float valve and quartz immersion heaters.
Drill 1 mm holes in microscope slides.
Design and fabricate water jacketed chromatograph column.
Fabricate radioautograph tube.
Design and fabricate quartz radiation vessels.
Fabricate 4 steam generators.
Develop and fabricate 6 orifice assemblies for Coulter Counter with 100 μ thick orifice.
Design and fabricate glass coil for disc electrophoresis tank.
Fabricate 4 quartz flow cells for spectrophotometer.
Fabricate multiple tooled heater tubes.
Fabricate 3 large lyophilizers.
Design and fabricate 9-liter tissue culture flasks.
Design and construct flow cell holders.
Design and develop Sephadex columns.
Design and fabricate timed automatic sample removing device.
Design and fabricate animal type holders and chambers for total body irradiation (mice).
Fabricate immunoelectrophoresis plates and holders.
Modify roller tube drum to operate at many reproducible speeds.
Design and fabricate stainless steel coupling unit for teflon culture spinner.
Construct adapters and various holders for X-ray units.
Fabricate plexiglas tank for X-ray dosage determinations.
Design and fabricate ion chambers and probes.
Design and fabricate graduated device to measure central axis distances for Co^{60} head.
Remodify Skinner Boxes to provide constant automation.
Fabricated electronic cell colony counter.
Constructed automatic alternator of solid-state elements to prevent transmitted pulse from overloading receiver in sonic probe device without desensitizing receiver to reflected signal.
Provided means of obtaining visible and punched tape information corresponding to physical activity of experimental animals.
Devised circuity to present digital information corresponding to physical activity of experimental animals.
Replaced electronic components and rewired Base-Compensating U-V analyzer.

The administrative staff more than usual performed a Herculean task. With the retirement of one member in October and sustained illness by another since early February, the two remaining members completed all the work of the office, accepting the challenge of insufficient help. Letters of commendation will be submitted for their personnel files.

Serial No. NCI-900
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies in Tumor-Host Relationships

Previous Serial Number: Same

Principal Investigator: Julius White

Man Years

Total:	4
Professional:	2
Others:	2

Project Description:

Objectives: To study the relationships between water, diet and salt intake on the growth of the Walker 256 carcinosarcoma.

Major Findings:

1. Rats bearing the Walker 256 tumor and fed a diet in which the source of protein is lyophilized Walker tumor grew larger tumor and gained more body weight (minus tumor), than did similar tumor-bearing animals ingesting a diet in which casein or casein supplemented with 0.35 or .70% NaCl.

2. Average daily water intake was also higher for animals on the tumor diet, being 40 per cent higher. Animals in the casein or casein salt diet all showed a remarkably equivalent water intake.

3. High concentrations of NaCl (.70%) resulted in smaller tumors and lowered body weight gains.

Significance to Bio-medical Research and the Program of the Institute: Electrolyte and H_2O exchange in tumor-bearing animals play an important role in tumor growth.

Proposed Course of Project: To continue this study.

Serial No. NCI-949
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Effects of a Transplanted Anterior Pituitary Tumor

Previous Serial Number: Same

Principal Investigators: Julius White and Florence K. Millar

Man Years

Total:	0
Professional:	0
Others:	0

Project Description:

The objectives, methods and findings of this project are the same as reported previously (NCI-949, 1966). No further experiments are anticipated. A report on the results of this investigation is in preparation.

Serial No. NCI-918
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Research Development Projects in the Field of Electronics.

Previous Serial Number: Same

Principal Investigator: Nathan Coffey

Other Investigator: William Comstock

Man Years

Total:	2
Professional:	2
Others:	0

Project Description:

Objectives: To design new electronic equipment as the needs of the Laboratory arise; to modify existing electronic equipment to meet the changes required in an experiment; to supervise any research development projects in the field of electronics; and to provide the facilities of the Mass Spectrometer to the Laboratory.

Major Findings: The following is a list of the major jobs in developmental electronics completed during the reporting period:

Provided electronic cell colony counter.

Devised and supplied several innovations to extend versatility of Skinner Boxes.

Constructed automatic attenuator of solid-state elements to prevent transmitted pulse from overloading receiver in sonic probe device without desensitizing receiver to reflected signal.

Provided means of obtaining visible and punched tape information from analog recorder on O_2 analyzer.

Devised circuitry to present digital information corresponding to physical activity of experimental animals.

Replaced electronic components and rewired Base-Compensating U-V Analyzer.

Processed more than 900 samples in the Mass Spectrometer, including:

N^{15} urine uric acid, lithium urate, ammonium sulphate and nitrogen, O_{18} mixture analysis, methane.

Processed prepared N^{15} samples on converter and prepared ammonium sulphite and sodium hypo-bromide for analysis.

Performed routine and preventive maintenance on electronic devices required in carrying out investigative programs - e.g., oscilloscopes, spectrophotometers, grandient analyzer, mass spectrometer, pH meters, electrometers, analog recorders, analog-to-digital converters, etc.

Significance to Bio-medical Research and the Program of the Institute: Provides capability of securing refined procedures for sensing and measuring physiological factors, and for recording pertinent data in a form most suitable for manipulation.

Proposed Course of Project: To continue to provide a facility for combining advanced electronic techniques with advanced procedures in physiological research to insure rapid and accurate collection of experimental data.

Serial No. NCI-947
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Development, Fabrication and Maintenance of Specialized Equipment for Laboratory of Physiology, Radiation Branch, and Several Other Laboratories in NCI.

Previous Serial Number: Same

Principal Investigators: Calvin R. Mencken and Edward J. LeBrun

Man Years

Total:	4
Professional:	2
Others:	2

Project Description:

Objectives: After discussion with the senior scientists in the NCI located in Bldg. 10, designs and fabricates instruments that are not available on the open market. Modification of existing equipment to increase its versatility, accuracy, and requirements are also performed. In addition, servicing and repairing heavy duty equipment such as Van de Graaff generators, X-ray equipment, mechanized automatic recording devices are undertaken. Examples of such performances are as follows:

Glass Shop - Physiology:

Fabricate 2 stage water still with automatic float valve and quartz immersion heaters.

Fabricate 50 irradiation vessels with two break-seals.

Drill 1mm holes in microscope slides.

Fabricate water jacketed chromatograph column.

Fabricate 12 urine separators.

Fabricate 1 radioautograph tube.

Develop quartz radiation vessels.

Fabricate 4 steam generators.

Develop and fabricate 6 orifice assemblies for Coulter Counter - size

of orifice 100μ thick.

Glass Shop - Dermatology:

Design and fabricate glass coil for disc electrophoresis tank.

Glass Shop - Biology:

Fabricate 2 chromatograph trays.

Fabricate 4 quartz flow cells for spectrophotometer.

Modify and fabricate 3 mixing chambers for gradient elutions.

Fabricate large ultra-filtration units.

Fabricate 200 tooled heater tubes.

Fabricate special suction needles.

Glass Shop - Immunology:

Fabricate 6 ultrafibration bulbs.

Fabricate 3 large lyophilizers.

Fabricate 1 manifold.

Glass Shop - Laboratory of Chemical Pharmacy:

Develop and fabricate 3 each 9-litre tissue culture flasks.

Fabricate glass aspirating manifold.

Total Jobs - 198.

Machine Shop - Laboratory of Physiology:

Design and construct flow cell holders.

Develop and construct holders and suction device for DNA extraction, for sucrose density grandient.

Design and fabricate flow cell holders.

Design and fabricate Sephardix Columns.

Design and fabricate timed automatic sample removing device.

Modify and rebuild syringe drive.

Fabricate various type holders and chambers to irradiate mice.

Machine Shop - Immunology Branch:

Develop and construct Filteration Columns.

Fabricate immunoelectropharesis plates and holders.

Fabricate incubation trays and humidifier chambers.

Machine Shop - Biology Branch:

Design and fabricate flow cell holder.

Modify roller tube drum to operate at many reproduceable speeds.

Design and fabricate stainless steel coupling unit for teflon culture spinner.

Machine Shop - Radiation Therapy:

Fabricate numerous lead shielding blocks of various shapes.

Construct adapters and various holders for Van Gard X-ray unit.

Repair extrapalatim chamber.

Fabricate plexiglas tank for X-ray dosage determinations.

Fabricate ion chambers and probes.

Design and fabricate graduated device to measure central axis distances for Co^{60} head.

Design and fabricate mounting bracket for TV camera.

Serial No. NCI-905
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Iodide-Concentrating Mechanism of the Thyroid Gland

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Other Investigator: Dr. George B. Eagleton

Man Years

Total:	1-2/3
Professional:	1-1/3
Others:	1/3

Project Description:

Objectives: To learn about (a) the mechanism by which the thyroid gland maintains a concentration of iodide elevated above that in the blood, and (b) the mechanism by which the thyroid gland accumulates protein-bound iodine.

Major Findings: In the hamster, subcellular organelles in the thyroid epithelial cell are stratified rather than being randomly arranged as in many other species.

Significance to Bio-medical Research and the Program of the Institute: This work is leading to an understanding of the relative importance of various factors in controlling the accumulation of radioisotopes by tissues.

Proposed Course of Project: To be continued.

Publications:

Andros, G., and Wollman, S. H.: Autoradiographic localization of radioiodide in the thyroid gland of the mouse. Am. J. Physiol. 213: 198-208, 1967.

Loewenstein, J. E., and Wollman, S. H.: Kinetics of equilibrium labeling of the rat thyroid gland with ^{125}I. Endocrinology 81: 1063-1073, 1967.

Loewenstein, J. E., and Wollman, S. H.: Distribution of organic ^{125}I in the rat thyroid gland during equilibrium labeling as determined by autoradiography. Endocrinology 81: 1074-1085, 1967.

Loewenstein, J. E., and Wollman, S. H.: Diffusion of thyroglobulin in the lumen of the rat thyroid follicle. Endocrinology 81: 1086-1090, 1967.

Serial No. NCI-906
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Production and Properties of Transplantable Thyroid Tumors

Previous Serial Number: Same

Principal Investigator: Seymour H. Wollman

Other Investigator: Dr. George B. Eagleton

Man Years

Total:	1-1/3
Professional:	1-1/3
Others:	0

Project Description:

Objectives: To study thyroid growth and the production and properties of transplantable thyroid tumors in the rat: changes occurring during growth, hyperplasia and involution, histologic types of tumors and their progression, and the correlation of histology with the biochemistry and physiology of the tumors.

Major Findings: Some follicles of the hyperplastic thyroid gland of the mouse are bordered by a double layer of epithelial cells. The true lumen of the follicle is between the two rows of cells. The inner layer of cells is polorized with microville extending into the true lumen, but no basement membrane at the basal end of the cells of the inner layer was observed.

Significance to Bio-medical Research and the Program of the Institute: These studies are giving a better idea of the conditions existing in the thyroid gland at the time tumors are induced. They also are giving information on the range of variation of properties of presumably normal cells.

Proposed Course of Project: To be continued.

Serial No. NCI-928
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Intracellular Colloid Droplets

Previous Serial Number: Same

Principal Investigators: Seymour H. Wollman
Dr. Ira Pastan

Cooperating Unit: Clinical Endocrinology Branch, NIAMD

Man Years
Total: 1/3
Professional: 1/3
Others: 0

Project Description:

Objectives: To study the properties of intracellular colloid droplets in the thyroid gland in order to decide whether they are newly formed colloid or colloid which has been resorbed from the lumen of the follicle.

Major Findings: We have observed that morphologic changes of dog thyroid gland characteristic of resorption of colloid and secretion of thyroxine, namely, induction of apical pseudopods and formation of intracellular colloid droplets can be induced in vitro by incubation of thyroid tissue slices with thyroid stimulating hormone. Such effects can be duplicated by incubation with dibutyryl cyclic adenosine monophosphate. This suggests that cyclic adenosine monophosphate may be an intracellular mediator of the effects of thyroid stimulating hormone.

Significance to Bio-medical Research and the Program of the Institute: This work may lead to a better understanding of the mechanism of hormone secretion and, hence, radioisotope release in the thyroid gland.

Proposed Course of Project: To be continued.

Publication:

Pastan, I., and Wollman, S. H.: Colloid droplet formation on dog thyroid in vitro. J. Cell Biology 35: 262-266, 1967.

Serial No. NCI-919
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Urinary Excretion Patterns in Leukemia

Previous Serial Number: Same

Principal Investigator: James C. Reid

Man Years

Total:	1.0
Professional:	1.0
Others:	0

Project Description:

Objectives: This program is a developmental effort to expand the scope of urinary analysis. Its objectives are the development of a methodology to broaden greatly the chemical information which can be extracted from the urine and the evaluation of such information for potential utility from both the clinical and research standpoints.

Experimentally, a search is being conducted for significant abnormalities in the excretion patterns of all detectable ultraviolet-absorbing components in a set of urine specimens collected from leukemic and control subjects. Such components include various nucleic acid congeners.

Methodology: The basic concept is that it may be feasible to construct an analytical system powerful enough to deal with the complexity of urinary composition by the application of a computational technique for multicomponent mixture analysis to urinary fractions obtained after preliminary chromatographic simplification. The data required are simply absorbance spectra which can be obtained by a single non-specific instrumental procedure. The necessity for the application of many chemical procedures, each specific for a particular component, is eliminated thereby and the attendant reduction of labor would make it feasible to study many components simultaneously, i.e., to study patterns of excretion. Moreover, techniques which have been developed in this program permit quantitative estimation of the components without the necessity for chemical identification, a feature which further facilitates the recognition of pattern abnormalities if they should occur.

The preliminary chromatographic simplification is effected by means of a system of ion-exchange column procedures developed earlier in the course of this program. The system reduces each urine specimen to approximately fifty

fractions. Each fraction is a mixture whose components typically number about a half-dozen, and in general the individual components can be isolated in small amounts by paper chromatography. The absorption spectra of isolated components can be obtained and then serve as a basis for computing the quantitative composition of the corresponding fraction obtained from other specimens of urine.

The intrinsic sensitivity of the methodology is high enough to deal with very low excretion levels.

Project Status:1. The principal experimental requirement at the present stage is the isolation of pure specimens of the components of the various urinary fractions and the construction of a reference file of their spectra to serve as a basis for computing mixture compositions. As during the preceding year, laboratory assistance lost earlier has not been replaced nor has programming assistance lost at the same time. Consequently, experimental work has remained in deferred status and project activity continues to center on a variety of computational problems associated with multicomponent analysis. The immediate objective is the standardization of the technique, i.e., the determination of the optimum mathematical formulation, the definition of its limits of applicability, and the development of technique for checking the validity of computed solutions.

2. The categorization which follows affords a brief indication of the character of the problems involved and of the programming employed in this investigation:

A. Numerical Precision - Loss of accuracy can occur on the subtraction of two numbers of similar magnitude and magnified by the occurrence of the difference as a divisor in subsequent stages of an extended computation. A program option permits variable perturbation of the input to evaluate the sensitivity of a computed solution to such effects.

B. Noise Analysis - Noise, i.e., normal error in the input data can be classified as random or biased. Random noise arises in the measuring instruments. It can be dealt with by making measurements at a large number of wavelengths and applying least squares reduction to such an "over-determined" system. However, it has been found that, with the instrumentation being used, the effect of random noise is minor relative to that of biased noise.

Biased noise arises from chemical factors such as blanks which may not be precisely compensated by the program which performs this function. Its effect is not exerted equally over all wavelengths, hence, an optimum subset of data points exists. The implication is that the use of a highly over-determined system is not the preferred mathematical formulation.

C. Input Optimization - The problem of finding the best set of wavelengths to use for a particular application is difficult to handle analytically, but several heuristic approaches have been devised, designed to allow the computer to select automatically wavelengths at which there is

an optimal relationship between the weighting of the noise, the component of interest and the characteristics of the associated matrix. The programming of these is still in progress.

D. Matrix Characteristics - A given set of component spectra has associated with it an algebraic matrix which possesses an intrinsic "quality". The quality of the matrix governs the quality of the ultimate output from the computation and is determined by factors such as the number of components and the degree of differentiation of their spectra. Numerical indexes of matrix quality can be computed and their empirical correlation with the quality of test output affords a rationale for estimating the feasibility of a particular analysis. Complete matrix analysis is incorporated into the programming; as well as facilities for generating computed test mixtures for as many as ten components of any specified composition.

E. A variety of other program features provide automatically for items such as data which are below a specified instrumental precision level and perform analysis of the final solution to determine its compatibility with the mixture data initially presented.

3. The programming is not yet in final form and systematic evaluation is not available. Fragmentary results obtained in connection with program test runs show good accuracy with three-component mixture data prepared artificially as mentioned in paragraph 2D even when the matrix quality is low. Larger systems require considerable running time and their investigation is being deferred until the programming has been fully checked and operating reliably.

Significance to Bio-medical Research and the Program of the Institute: One of the physician's major sources of information concerning the clinical status of a patient is urinalysis. Viewed as an information file the content of urine is very great, yet comparatively little of it is available routinely at present. This project is an attempt to make more of it available and to evaluate its potential utility.

In addition to the clinical aspect such information is potentially useful in the research context in understanding the action of antileukemic drugs and the nature of the disease. Because of generality of the methodology, however, potential applications are not confined to the cancer area.

One of the components of the methodology, namely multicomponent mixture analysis, has broad potentials in analytical chemistry, where it can be applied to data from many types of spectrometers. It may simplify and speed up some analytical procedures by reducing the necessity for preliminary isolation steps. In particular, it may be useful in special applications such as investigation of the kinetics of fast reactions where conventional methods cannot be applied.

Serial No. NCI-925
1. Laboratory of Physiology
2. Cancer Physiology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Protein Synthesis and Its Control in Normal and Tumor Cells

Previous Serial Number: Same

Principal Investigator: Marco Rabinovitz

Other Investigators: Michael L. Freedman, (Mark E. Smulson and T. R. Breitman for part of work as indicated)

Man Years

Total:	4.5
Professional:	3.0
Others:	1.5

Project Description:

Objectives:

a) To study, in both normal and tumor cells, the route or routes taken by amino acids in the synthesis of protein, and the controls exercised by the cells in regulating these processes. This is generally made through an analysis of the biochemical lesions introduced within the pathway by inhibitors of protein synthesis.

b) Where applicable, to extend the information obtained in the first objective to the tumor-bearing animal with the view of selective inhibition of protein synthesis in cancer cells.

Methods Employed:

a) Organic synthetic techniques are developed for preparation of potentially effective amino acid antagonists.

b) Incorporation of carbon-14 or tritium labeled amino acids into protein of isolated cells is measured. Such studies involve radioactive counting techniques and stripping film radioautography. Respiration and anaerobic glycolysis are followed by standard manometric techniques.

c) Subcellular components are isolated by differential and zonal centrifugation. These components are further fractionated by extraction, hydrolysis or chromatographic techniques. The characteristics of the block in amino acid utilization within these fractions are evaluated.

Major Findings:

1. Polyribosome Structure Related to Inhibition of Protein Synthesis at Specific Loci During Assembly of the Hemoglobin Molecule.

Specific amino acid deficiencies and antagonisms promote characteristic changes in the polyribosome structure of intact rabbit reticulocytes. These changes are readily reversible upon addition of the corresponding metabolite. Omission of tryptophan or interference in the utilization of methionine with α-methylmethionine causes polyribosome disaggregation. Both tryptophan and methionine are located only near the amino-terminal end of both globin chains, hence ribosomes are retarded in movement at the corresponding codons near the proximal end of mRNA. A single cysteine residue is located closer to the distal portion of mRNA for both α- and β-chains, and a deficiency of cysteinyl-tRNA brought about by α-methylcysteine causes the formation of larger polyribosomes. Isoleucine is located near the proximal end of the α-chain and near the distal end of the β-chain. The isoleucine antagonist, O-methylthreonine, causes an increase in small α-chain polyribosomes (dimers and trimers) and the formation of very large β-chain polyribosomes (duodecamers).

The relative stability of these large polyribosomes to the action of puromycin in the intact cell, and to proteolysis by pronase in isolated preparations, suggests that these polyribosomes are not formed by interaction of smaller polyribosomes through their nascent protein chains. The results indicate that interference with completion or release of protein from synthetic sites will induce the formation of large polyribosomes due to closer packing of ribosomes on a stretched mRNA.

Puromycin, by substituting for tryptophanyl-tRNA, will rectify the disaggregation of polyribosomes brought about by tryptophan deficiency. The antibiotic, however, will not cause reaggregation of polyribosomes which were disaggregated as a result of hemin or methionine deficiencies.

2. Membranes in Polyribosome Formation

The observation that tryptamine, a tryptophan analog, causes polyribosome disaggregation in reticulocytes, led to a study of derivatives of tryptamine. It soon became apparent that the required structure for this effect was not that of a tryptophan antagonist, but resided in several amino indoles. Primaquine, a structurally related amino quinoline, was also effective. This antimalarial is known to promote K^+ efflux from erythrocytes. Butanol, a model compound which causes reversible membrane damage and K^+ efflux in erythrocytes was therefore also investigated. This alcohol was found to cause a rapid polyribosome disaggregation which is dependent on protein synthesis. Dilution of the butanol below its critical effective concentration will induce the rapid reformation of polyribosomes. The reformation is also dependent on protein synthesis. This effect can be seen before any observable changes in the ATP of the cells or of K^+ efflux

is apparent, and is interpreted as indicating a sensitive membrane function in polyribosome formation.

3. Selective Inhibition of the Synthesis of α- and β-chains of Hemoglobin

Some hemoglobins contain certain amino acids in one chain but not in the other. By interference with utilization of this amino acid it is possible to investigate whether the chain in which the amino acid is absent is also synthesized. Human fetal hemoglobin has several isoleucine residues in the γ-chain but none in the α-chain. Dr. George R. Honig, a former research associate in this laboratory and now a resident in pediatric hematology, has investigated this system with reticulocytes of newborn infants. He found that the α-chain is synthesized when formation of the γ-chain is inhibited by L-O-methylthreonine. Suitable applications of this principle to other species should permit investigation of the inverse relationship and a check on its universality.

4. O-Methylthreonine Inhibition of Growth and of Threonine Deaminase in Escherichia coli (with M. E. Smulson and T. R. Breitman)

O-Methylthreonine, an isosteric analog of isoleucine, markedly inhibited growth of Escherichia coli 15. This inhibition was overcome most effectively by addition of isoleucine, valine, or leucine to the medium and less effectively by addition of threonine. The dipeptide, valyl-leucine, also relieved the inhibition but only after a lag period, suggesting that valine and leucine, liberated by dipeptidase action, compete with the analog for entry into the cell. O-methylthreonine was activated and transferred to transfer ribonucleic acid by isoleucyl-RNA synthetase in vitro. The rate of incorporation of the analog into protein of bacteria was comparable to that of isoleucine. Very high concentrations of O-methylthreonine were required to inhibit threonine deaminase, and the inhibition was strictly competitive with threonine. In addition, the analog inhibited a threonine deaminase preparation desensitized to isoleucine inhibition. In contrast to the results with E. coli, O-methylthreonine was not incorporated into the isoleucine-tRNA of liver, reticulocyte or sarcoma-37 ascites tumor.

Significance to Bio-medical Research and the Program of the Institute: These studies utilize nutritional deficiencies, antibiotics and other inhibitors as a means of comparing the process of protein synthesis and its control by normal and tumor cells. Such comparisons should furnish basic data of potential use for selective inhibition of cell types and specific protein-forming systems.

Proposed Course of Project: I hope to continue studies on the mechanism of action of hemin in polyribosome formation and to investigate problems of α- and β-chain synthetic synchrony. In addition, problems on the nature of the required metabolic status in organisms to produce a lethal effect by amino acid antagonists will be explored.

Publications:

Freedman, M. L., Hori, M., and Rabinovitz, M.: Membranes in polyribosome formation by rabbit reticulocytes. Science 157: 323-325, 1967.

Waxman, H. S., Freedman, M. L., and Rabinovitz, M.: Studies with ^{59}Fe-labeled hemin on the control of polyribosome formation in rabbit reticulocytes. Biochim. Biophys. Acta 145: 353-360, 1967.

Smulson, M. E., Rabinovitz, M., and Breitman, T. R.: O-methylthreonine inhibition of growth and of threonine deaminase in Escherichia coli. J. Bacteriol. 94: 1890-1895, 1967.

Freedman, M. L., Fisher, J. M., and Rabinovitz, M.: Puromycin interference of reticulocyte polyribosome disaggregation caused by tryptophan deficiency. J. Mol. Biol. (March 1968 issue)

Hori, M., and Rabinovitz, M.: Polyribosome structure during inhibition of hemoglobin synthesis by an isoleucine antagonist. Proc. Natl. Acad. Sci., U. S. (April 1968 issue)

Smulson, M. E., and Rabinovitz, M.: L-O-methylthreonine-synthesis and mode of action as an isoleucine antagonist. Arch. Biochem. Biophys. 124: 306-313, 1968.

Serial No. NCI-916
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Characterization and Comparison of the Nucleic Acids, Nucleoproteins and Related Components of Normal and Malignant Tissues

Previous Serial Number: Same

Principal Investigator: Joseph Shack

Man Years

Total:	3
Professional:	3
Others:	0

Project Description:

Objectives:

1) Characterization of the nucleic acids, nucleoproteins and certain other related substances in normal and malignant tissues in terms of physical, chemical and biological properties. Within this framework specific examples of experimental objectives include determination of a) whether the DNA or nucleoproteins of malignant cells differ from that of their normal counterparts by, for example, deletion or change of normal components or addition of abnormal ones; b) whether proteins of particular types, e. g., histones of the arginine or lysine type, or non-histone protein, are preferentially associated with DNA segments of various types (such as low or high guanine-cytosine, repetitive or non-repetitive); c) how the DNA-protein relationship changes during the division of the cell, particularly during and following the period of DNA replication; d) whether the DNase inhibitor present in such great amount in human white cells might provide a useful index in relation to diseases involving white cells.

2) Development of new procedures for isolation, fractionation and characterization of the types of nucleoproteins discussed in Part 1) above.

Major Findings: Particular attention was given, in the early portion of the year, to identification of a factor in human serum which stabilizes the DNase inhibitor prepared from human white blood cells. It was extensively purified through a combination of preparative electrophoresis and zonal sedimentation and identified as an α_1 globulin with a low (ca. 4) sedimentation constant.

Following development of procedures for estimation of both DNase and DNase inhibitor in urine, a number of urines from leukemic subjects were tested for presence of inhibitor. As with normal urine, no significant amount of inhibitor was found in the specimens tested to date.

Using cells grown in tissue culture as a source of material, it was found, by a combination of pulse labelling and electrophoretic analysis, that the nucleoprotein containing newly-formed DNA can be distinguished from, and at least partially separated from, the bulk of the nucleoprotein component.

Significance to Bio-medical Research and the Program of the Institute: This project has been in existence since 1948. At that time the phenomenon of bacterial transformation was the principal reason for our belief that research on nucleic acids would make major contributions to an understanding of malignancy. Since then, numerous developments in biology and biochemistry have strongly supported this assumption.

Recent developments that focus on nucleoproteins are the various lines of evidence that suggest that histones are of importance in the regulation of the expression of genetic information encoded in the DNA component. Our interest in DNase inhibitor is due in large part to the fact that the amount of inhibitor per average white cell is much larger than in any other type of cell and it seemed reasonable that knowledge of this substance might be useful in connection with various diseases involving white cells, such as the leukemias.

Proposed Course of Project: Work will continue along the same lines. It is at present expected that a major portion of the work will be concerned with the physicochemical and metabolic properties of the intact deoxyribonucleoproteins of mammalian tumor cells.

Serial No. NCI-948
1. Laboratory of Physiology
2. Physical Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Control of Ribo- and Deoxyribonucleotide Synthesis

Previous Serial Number: SAME

Principal Investigator: T. R. Breitman

Man Years

Total:	3.5
Professional:	3.0
Others:	0.5

Project Description:

Objectives: To clarify control mechanisms of purine and pyrimidine ribo- and deoxyribonucleotide biosynthesis with special emphasis on the role of thymine deoxyribonucleotides.

Major Findings:

A. Control of Deoxyribonucleotide Biosynthesis in Growing Escherichia coli.

Our previous study (W. D. Cannon, Jr., and T. R. Breitman, Biochemistry 6: 810, 1967) showed that when E. coli is grown in the presence of deoxythymidine 5'-monophosphate (dTMP), a 50% increase of the thymine deoxynucleotide pool is associated with a decrease of pyrimidine deoxyribonucleotide biosynthesis de novo, a 30% decrease of the cytosine and uracil deoxyribonucleotide pools and a 37% decrease of the contribution de novo to the thymine deoxyribonucleotide pool though a distinction could not be made between feedback control and/or repression. The studies of Reichard and his collaborators showed that the substrate specificity of E. coli ribonucleotide reductase is subject to allosteric control by ATP and deoxyribonucleoside triphosphates. Based on these studies in vitro, a model was formulated which could be used as a guide to the situation in the intact cell. One aspect of this model particularly pertinent to our own study was that an intracellular concentration of deoxythymidine triphosphate that inhibits synthesis of pyrimidine deoxyribonucleotide might enhance synthesis of purine deoxyribonucleotide. Our present investigation was initiated in order to estimate the effect of an increased intracellular thymine deoxyribonucleotide pool size on purine deoxyribonucleotide biosynthesis and to access if changes in deoxyribonucleotide biosynthesis resulted from feedback inhibition and/or a change in the level of ribonucleotide reductase.

Growth of E. coli in the presence of 10^{-4} M dTMP resulted in a 40% increase of the total thymine deoxyribonucleotide pool. In addition, there were (a) approximately 40% decreases of the uracil and cytosine deoxyribonucleotide pools, and of the contribution de novo to the thymine deoxyribonucleotide pool, (b) no decreases of the pyrimidine ribonucleotide pools, (c) no significant changes on the purine deoxyribonucleotide pools, and (d) no change in ribonucleotide reductase activity assayed in crude extracts. These results indicate that the decrease in pyrimidine deoxyribonucleotide biosynthesis is due to feedback inhibition rather than repression of ribonucleotide reductase.

B. Studies on the Metabolism of Thymineless Mutants of Escherichia coli.

In a previous communication (T. R. Breitman and R. M. Bradford, Biochim. Biophys. Acta 138: 217, 1967) we reported that E. coli 70V3, a low thymine-requiring mutant of E. coli 15, lacked deoxyriboaldolase activity, establishing a biochemical basis for the low requirement. Also presented was data indicating that both deoxythymidine phosphorylase and deoxyriboaldolase were induced coordinately when E. coli was grown on deoxythymidine and that deoxyribose-5-phosphate was directly involved with these inductions.

We have subsequently investigated other low thymine-requiring mutants derived from E. coli 70 (a high thymine-requiring mutant derived from strain 15) and found that some of these strains lacked phosphodeoxyribomutase and are therefore unable to synthesize deoxyribose-5-phosphate from deoxythymidine. In these strains, growth on deoxythymidine did not result in the induction of either deoxythymidine phosphorylase or deoxyriboaldolase establishing that the production of deoxyribose-5-phosphate is required for induction.

C. Studies on the Synthesis of Phage-specific Enzymes.

In collaboration with Drs. R. C. Shuster and A. Weissbach (NIAMD) a study was initiated to determine if the synthesis of a phage-specific deoxyribonuclease and DNA polymerase (enzymes known to appear early in the growth cycle of coliphage λ and several other temperate inducible coliphages) were also associated with the production of all temperate coliphages. Of particular interest was the study of strain 15, which carries a defective prophage. The results showed that under conditions of phage induction in strain 15, as well as two other phage strains, there was no synthesis of either deoxyribonuclease or DNA polymerase. The findings with strain 15 represented the first example of an inducible temperate phage lacking the new nuclease.

D. Studies on Human Leukocytes

In collaboration with Drs. R. C. Gallo and S. Perry, NCI, studies on pyrimidine metabolism in human leukocytes, reported on previously in the 1966 Annual Report have continued.

It was found that the synthesis of deoxythymidine (dThd) from thymine in cell-free systems prepared from human leukocytes (normal and chronic myelogenous leukemic) is mediated by two enzymatic mechanisms, pyrimidine deoxyribosyl transferase and deoxythymidine phosphorylase. Both activities are probably the function of one protein.

dThd synthesis by either mechanism is inhibited by thymine. This inhibition by thymine of pyrimidine deoxyribosyltransferase is competitive with the deoxyribosyl donor, while inhibition of deoxythymidine phosphorylase is of a mixed type, resulting in both a decrease in the Vmax and an increase in the Km for deoxyribose 1-phosphate. Similarly, uracil inhibition of deoxyuridine synthesis by the phosphorylase mechanism is of a mixed type. However, there is essentially no inhibition by uracil of deoxyuridine synthesis mediated by pyrimidine deoxyribosyl transferase.

Pyrimidine deoxyribosyl transferase is activated by inorganic arsenate or phosphate. This activation is not due to an increase in the affinity of the enzyme for either deoxyuridine or thymine, nor is it due to a shift in the pH optimum.

The enzymatic mechanisms for dThd synthesis are, therefore, influenced by modifiers, a finding suggesting that these activities may play a significant role in the metabolism of deoxythymidine and deoxyuridine in the intact mammalian cell.

The findings discussed above prompted an investigation of the relative efficiencies of various deoxyribosyl donors to support the synthesis of dThd from thymine in crude leukocyte extracts. It was found that synthesis of dThd is catalyzed from a deoxyribonucleoside to thymine (pyrimidine deoxyribosyltransferase) and by a coupled deoxynucleoside phosphorylase mechanism in which dThd is synthesized from deoxyribose 1-phosphate formed after phosphorolytic cleavage of either purine or pyrimidine deoxynucleosides. Both enzymatic mechanisms participate in dThd synthesis with pyrimidine deoxynucleosides as the deoxyribosyl donor. However, the proportion synthesized by either mechanism is dependent on the concentration of deoxynucleoside. At concentrations above 5 mM the transferase is the predominant enzymatic mechanism.

Purine deoxynucleosides, on the other hand, participate in dThd synthesis only by a coupled deoxythymidine phosphorylase mechanism. Rates of dThd synthesis with purine deoxynucleosides as deoxyribosyl donors were approximately 1/10 to 1/30 of the rates with pyrimidine deoxynucleosides.

The constant ratio of transferase to phosphorylase through a 140-fold purification and during heat inactivation studies suggest that both activities are the function of one protein. In the presence of thymine and both deoxyuridine and deoxyribose 1-phosphate, synthesis of dThd occurs independently by both enzymatic mechanisms, indicating that there are separate sites for each deoxyribosyl donor.

The finding, described above, that purine deoxyribonucleosides inefficiently support dThd synthesis from thymine led to an investigation that showed that purine bases inhibited deoxythymidine phosphorylase in the direction of dThd synthesis. The most effective inhibitors are the 6-oxypurines: hypoxanthine, xanthine, and allopurinol. 6-Mercaptopurine is also a potent inhibitor. The inhibition with a partially purified enzyme is characterized by a decrease in the Vmax for deoxyribose 1-phosphate with no change in the Km. Increasing concentrations of the second substrate, thymine, do not diminish the inhibition, however, analysis is complicated by substrate inhibition by thymine. Treatment of the enzyme with thymine prior to the addition of purines greatly diminished the inhibitory effects of purines. Attempts to selectively modify the inhibitor sites with urea, mercury, heat, trypsin, or enzyme aging were unsuccessful. In the presence of urea the enzyme is less sensitive to thymine inhibition, but there is no decrease in purine inhibition. In addition, with increasing concentrations of urea there is a corresponding increase in the apparent Km for thymine.

Uridine phosphorylase, catalyzing uridine synthesis from uracil and ribose 1-phosphate is inhibited by 6-mercaptopurine and hypoxanthine but not by allopurinol. dThd synthesis by direct transfer of deoxyribosyl from deoxyuridine to thymine (pyrimidine deoxyribosyl transferase) is also inhibited by purines, but the inhibition is slight compared to the inhibition of dThd synthesis from thymine and deoxyribose 1-phosphate.

Significance to Bio-medical Research and the Program of the Institute: Findings pertaining to an elucidation of mechanisms involved in the metabolism of nucleic acid components are important as they may suggest possible means of therapeutic control of cellular growth.

Proposed Course of Project:

1. An *in vivo* study in *E. coli* of the effects of a decreased thymine deoxynucleotide pool on the synthesis of the other deoxyribonucleotides.

2. Initiate a study of the metabolism of 5-ribosyluracil (pseudouridine) in *E. coli*.

Publications:

Shuster, R. C., Breitman, T. R., and Weissbach, A.: The failure of three temperate coliphages to direct the synthesis of a new λ type deoxyribonuclease. J. Biol. Chem. 242: 3723-3725, 1967.

Gallo, R. C., Perry, S., and Breitman, T. R.: The enzymatic mechanisms for deoxythymidine synthesis in human leukocytes I. Substrate inhibition by thymine and activation by phosphate or arsenate. J. Biol. Chem. 242: 5059-5068, 1967.

Smulson, M. E., Rabinovitz, M., and Breitman, T. R.: O-Methylthreonine inhibition of growth and of threonine deaminase in Escherichia coli. J. Bacteriol. 94: 1890-1895, 1967.

Cannon, W. D., Jr., and Breitman, T. R.: Control of deoxynucleotide biosynthesis in Escherichia coli II. Effect of deoxythymidylate on the level of deoxynucleotides and ribonucleotide reductase. Arch. Biochem. Biophys., in press.

Breitman, T. R., and Bradford, R. M.: The inability of low thymine-requiring mutants of Escherichia coli lacking phosphodeoxyribomutase to be induced for deoxythymidine phosphorylase and deoxyriboaldolase. J. Bacteriol, in press.

Serial No. NCI-945
1. Laboratory of Physiology
2. Office of the Chief
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of the Factors Regulating the Initiation of Specific Protein Synthesis During Embryonic Development

Previous Serial Number: None

Principal Investigator: Beverly Peterkofsky

Man Years

Total:	1
Professional:	1
Others:	0

Project Description:

Objectives: To uncover the factors responsible for initiating and controlling the synthesis of tryptophan pyrrolase, an hepatic enzyme and collagen, a structural protein in embryonic tissues.

Major Findings: The present phase of this project is concerned with developing methods for detecting small amounts of these proteins.

a. A sensitive new assay has been devised for tryptophan pyrrolase based on the release of radioactive formic acid from 2-C^{14}-L-tryptophan through the sequential action of the enzymes tryptophan pyrrolase and formyl-L-kynurenine formylase. This assay is being used to analyze chick embryos at various stages of development for the presence of tryptophan pyrrolase. The effect of glucocorticord hormones on the developmental pattern of this enzyme will also be studied since these hormones are known to affect the rate of synthesis of the enzyme in adult liver tissue.

b. Collagenase, an enzyme which specifically hydrolyzes certain peptide bonds in collagen, will be used in a sensitive new assay for this structural protein. Partially purified collagenase is available commercially but it has been found that this preparation must be further purified to remove small amounts of non-specific proteolytic enzymes which interfere in the assay.

Significance to Bio-medical Research and the Program of the Institute: The study should elucidate some of the regulatory mechanisms operative in normal rapidly proliferating embryonic cells which result in differentiated cells carrying out specific functions. When such normal mechanisms are understood we can have a standard of reference for detecting abnormalities occurring at this level in malignant cells.

Proposed Course of the Project:

1. The effects of various compounds, such as glucocorticoid hormones, on the normal pattern of tryptophan pyrrolase accumulation in chick embryos will be investigated further.

2. Purified collagenase will be utilized in an assay for detecting small amounts of radioactively labeled collagen. The rate of synthesis of collagen in intact chick embryos will be determined using this assay and the factors responsible for initiating or altering the rate of synthesis will be analyzed using a cell-free system capable of synthesizing this protein specifically.

Serial No. NCI-944(a)
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Total Metabolism

Previous Serial Number: Same

Principal Investigators: Seoras D. Morrison and Florence K. Millar

Man Years

Total:	6.0
Professional:	1.5
Others:	4.5

Project Description:

Objectives:

(a) Investigation of the patterns of heat production of rats with concomitant recording of feeding behavior and other activity with a view to finding how the energy expenditure, feeding and general behavior pattern are related to tumor growth and to finding the nature of the loads imposed by a tumor on the host.

(b) Investigation of the relative changes in water and material exchanges and in tissue compartments of the rat during imposed and induced changes in food and water intake and the relation of these to tumor induction and growth.

(c) Construction of a model of the growth of the Walker 256 tumor.

Methods Employed: The methods of indirect calorimetry, operant conditional response, placement of electrolytic lesions in the central nervous system and the methods of orthodox nutritional studies (see NCI-944(e), 1962). Computer methods of numerical analysis of serial records of tumor growth. Sprague-Dawley rats were used in all animal experiments. All tumor-bearing rats were inoculated with Walker 256 carcinosarcoma.

Major Findings:

(a) The activity compartment of energy expenditure in normal rats and the modification of this compartment in rats made aphagic by hypothalamic lesions have been defined in detail (NCI-944(a), 1967). Full reports of this work are now in press. Application of these results to partition of energy expenditure in the tumor-bearer and the analysis of the loads imposed by a tumor on the host (NCI-944(a), 1967) has been continued.

There are three and possibly four compartments of energy expenditure in tumor-bearing rats; the energy expenditure of the tumor, the energy expenditure of the host, an energy compartment generated by the tumor but expended by the host and an increment of expenditure of the tumor induced by its presence in the host. The last two compartments limit the endogenous expenditure, physical activity and food intake of the host and correspond, approximately, to the energy equivalent of the lactic acid production of the tumor. Work is in progress to find whether continuous perfusion with equivalent amounts of lactic acid impose similar constraints on the physical activity and food intake of normal rats.

(b) The sustained or increased water intake of tumor-bearing rats in the presence of a declining food intake has been examined. Much of this water intake appears to be an indirect consequence of the reduced food intake if the reduced food intake is considered as a physiological food restriction. There is, however, a significant moiety of increased water intake attributable to some other effect of the tumor and this persists even in rats whose normal mechanisms of water balance have been disrupted by lateral hypothalamic lesions.

A full report of the relationship between water intake and work load (NCI-944(a), 1967) has now been published. The bearing of this on the water intake and depressed work potential of tumor-bearers is being pursued.

By measurement of the drinking responses to various stimuli in various experimental situations ("normal", recovered lateral, self-intra-gastric watering) it seems possible to identify some of the various mechanisms that regulate water intake. It appears that all of these mechanisms may not exist in all normal rats. It is hoped that continued work along this line may throw further light on the reasons for the anomalous features of water exchange during tumor growth.

(c) The lack of knowledge of the metabolizing mass of a tumor at any stage of its growth is a serious obstacle to partitioning the energy expenditure of a tumor-bearing animal (NCI-944(a), 1967). An attempt is being made to construct a model of growth of the Walker tumor so that its metabolizing mass at any time can be predicted *in vivo* from its total growth form. Some improvement in prediction has been achieved but some initial assumptions on the generation of viable and necrotic mass do not appear to be tenable. Experimental and computational work on this problem is continuing.

Significance to Bio-medical Research and the Program of the Institute: The findings on partition of energy expenditure and of tissue compartments and on processes influencing food and water intake make it possible to examine some features of tumor-host relationships which have previously not been susceptible to analysis. It is believed that this may throw useful light on the reasons for the rapid and extreme debilitation of the host during tumor growth.

Proposed Course of Project: Work on energy and water exchange of tumor-bearers will be continued with particular reference to the reasons for the changes in food and water intake and depletion of host tissues that occur during tumor growth.

Publications:

Morrison, S. D.: Regulation of water intake by rats deprived of food. Physiol. Behav. 3: 75-81, 1968.

Millar, F. K. and Morrison, S. D.: Relation of tissue electrolyte losses to the relative polydipsia of early starvation in rats. J. Nutr. 94: 211-218, 1968.

Morrison, S. D.: The constancy of the energy expended by rats on spontaneous activity, and the distribution of activity between feeding and non-feeding. J. Physiol. (Lond.), in press, 1968.

Morrison, S. D.: The relationship of energy expenditure and spontaneous activity to the aphagia of rats with lesions in the lateral hypothalamus. J. Physiol. (Lond.), in press, 1968.

Serial No. NCI-944(b)
1. Laboratory of Physiology
2. Energy Metabolism Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mathematical Programming

Previous Serial Number: Same

Principal Investigator: Florence K. Millar

Man Years

Total:	0.5
Professional:	0.5
Others:	0

Project Description:

Objectives: To develop mathematical and computer programming techniques for application in the investigation of selected bio-medical problems. For these activities, the Laboratory maintains an IBM 1620 data processing system which is available to the NCI staff. Use is also made of the central facility in the Computation and Data Processing Branch of the Division of Computer Research and Technology.

Some of the projects reported here have been undertaken as collaborative efforts with investigators in other organizational units of NCI.

Major Findings:

Computer Operations

The IBM 1620 Data Processing System with 40K digits of memory, 3 disk storage drives and a line printer is operated on a one-shift basis with the prime time shared by Drs. Reid and Millar and with occasional use by other scientific investigators of the NCI (see below). Computer operations include the procurement of the necessary supplies for maintenance of the computer hardware, scheduling the available time and maintenance, and making additions to the system software to handle data processing problems as they arise in the research of NCI scientists.

Computer Applications

a) Processing of raw data

(1) Processing of biochemical data from Tri-Carb liquid scintillation spectrometers [Drs. Breitman, Gallo (M), Mushinski (LBGY), Peterkofsky,

Scher; Mrs. Toal].

Several programs are in use for averaging the counts per minute from recycled samples, subtracting background, converting to disintegrations per minute, calculating statistical information, summing groups of samples, and quantitating the results from known specific activities. These programs include the capability for handling doubly labelling compounds.

(2) Analysis of immunological data [Dr. Draper].

A program calculates the daily output of antibody using known values for titer and decay rates. These and other data (e.g., antibody activity vs. radiation dose) are subjected to various statistical analyses including distribution characteristics, linear and nonlinear regression, correlation, t-test.

(3) Statistical analysis of radiation data [Drs. Caper, Fred, Withers].

Programs have been developed and are in use which carry out statistical analyses of data from radiation experiments (e.g., transform raw data as needed, calculate weighted means from pooled experiments, calculate and use dose vs. recovery curves).

(4) Processing of data from the small animal calorimeter [Dr. Morrison].

A start has been made toward checking and calculating the digital punched paper tape output from the analog-digital converter of the calorimeter system. The O_2 readings are converted to a format compatible with a Moseley X-Y Recorder for automatic plotting; thus it is possible to make a direct comparison between the analog O_2 trace and the digital read-out. Work is in progress to convert the instantaneous O_2 readings into a "square wave" form.

(5) Statistical treatment of enzyme kinetics [Dr. Lazafus (CH)].

A program has been developed and used to analyze results from enzyme reaction studies.

b) Information storage and retrieval

No further work has been done on the data from the chorio-carcinoma chemotherapy study.

c) Mathematical computation and model building

(1) Tumor growth model [Dr. Morrison].

Attempts are being made to estimate the metabolizing mass of a

subcutaneously transplanted tumor [see NCI-944(a), 1968, Major Findings, para (c)] by analysis of its day-to-day growth pattern (linear dimensions measured in vivo). Such techniques as the refinement, smoothing and plotting of the raw data, least squares fitting of derived data to a mathematical function, e.g., the Gompertz equation (A. C. Burton, Growth 30: 157, 1966) and testing of results predicted by theoretical solutions are accomplished through linked computer programs.

(2) Variable gradient curve formulation [Drs. Himmelhoch and Sober (LB), with the cooperation of Dr. S. A. Boorstein, Assoc. Prof. of Chemical Physics, Univ. of Calif. at Riverside].

The Varigrad (E. A. Peterson and H. A. Sober, Analyt. Chem. 31: 857, 1959) is a device for producing a variable gradient for chromatographic elution; it consists of serially linked chambers containing eluent of varying concentration. The shape of the elution gradient can be predicted mathematically from the initial concentrations in the chambers. The inverse problem, i.e., prediction of the initial concentrations required to produce a gradient curve of desired shape, has been approached by computer handling of the mathematical relationship involved. Both least squares fit and linear programming have been tried; the latter appears to be more useful but has yet to be tested on complicated systems.

(3) UV spectrophotometric analysis of oligoribonucleotides [Mrs. Toal].

This project was concluded in the 1966-67 period (See Part B of this report).

Significance to Bio-medical Research and the Program of the Institute: Mathematical and computer programming techniques contribute to the investigation of bio-medical problems by providing (1) accurate and efficient methods to handle laborious calculations of data, (2) techniques for storage and retrieval of large amounts of data, and (3) the ability to build and test mathematical models based on experimental results.

Proposed Course of Study: Computational techniques and programs will continue to be developed and applied as computer-related problems arise in the work of the NCI staff. In particular, work will continue on the tumor growth model and on the problem of reduction of digital data from the calorimeter.

Publications:

Toal, J. N., Rushizky, G. W., Pratt, A. W., and Sober, H. A.: Computer assisted characterization of oligoribonucleotides by their ultraviolet absorption. Analyt. Biochem. 23 (April 1968).

Tables from the above paper were selected for publication in the Handbook of Biochemistry, Chemical Rubber Publishing Co., 1968 (in press).

Serial No. NCI-933
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mechanisms of Action of Chemotherapeutic and Carcinogenic Drugs

Previous Serial Number: Same

Principal Investigator: M. M. Elkind

Other Investigators: Francesco Mauro and E. Kano, Research Associates; K. Sakamoto, Guest Worker.

Man Years

Total:	5.0
Professional:	3.5
Others:	1.5

Project Description:

Objectives: Mammalian cells can be killed by many agents. In the area of cancer research, two agents of interest are chemotherapeutic drugs and radiation. Further, both types of agents are capable of being mutagens and carcinogens. Since some chemical agents are radiomimetic, and since the cell biology involved in terms of modes of action may therefore be similar, a search for reparative systems in cells with respect to drug action is indicated. In addition, drug effects on survival and growth will also be studied.

Methods Employed: Using cultured mammalian cells and the general techniques used in radiobiological studies, we will examine colony formation changes, effects on population growth, and biochemical effects.

Major Findings: During the past year, additional progress has been made with actinomycin D and sulfur mustard, and in addition a good deal has been learned about nitrogen mustard:

Actinomycin D - As a required base line measurement for toxicity studies, we measured the rate of incorporation and retention of actinomycin D by mammalian cells using H^3 labelled drugs. We found that: 1) incorporation is linear with time and concentration over fairly broad limits; and 2) the half retention time of the drug is about 2-1/2 hours. This latter datum means that the effects of this drug are prolonged if only because of its binding within the cell. A further implication, which we confirmed experimentally, is that the toxic effect of the drug depends upon the number of cells present as well as the rinsing schedule. For a given surviving fraction, a larger

total number of cells means that a larger amount of drug is taken up and then released. As a result, the concentration of actinomycin D may build up to toxic levels in time even though otherwise adequate rinsing may have been employed.

Sulfur Mustard - The bifunctional sulfur mustard, $S(C_2H_4Cl)_2$, kills mammalian cells according to a sigmoid survival curve. As for X-rays, damage is accumulated before cells are killed. Further, as for X-rays, this sublethal damage is repairable as can be demonstrated using the two-dose technique. In spite of these similarities, different phases of the cell cycle are resistant to X-rays or sulfur mustard (i.e., late S for X-rays and G_2 for sulfur mustard).

Nitrogen Mustard - In contrast to our findings with sulfur mustard, the bifunctional agent $CH_3N(C_2H_4Cl)_2$ produces a survival curve which has a very small shoulder and hence, a small requirement for damage accumulation for lethality. This is true for asynchronous as well as synchronized cells examined in their resistant phase (S). Consistent with the absence of a requirement for sublethal damage, we found that essentially no repair of nitrogen mustard damage could be demonstrated using the two-dose technique. In addition, a study of the combined effects of X-rays and nitrogen mustard yielded the result that these agents are additive only (i.e., they do not interact).

Significance to Bio-medical Research and the Program of the Institute: A rational basis for the use of drugs in chemotherapy requires that their effects on cells be well understood. This is true for drugs used by themselves or in combination with radiation. A similar view is pertinent to carcinogens. In all these connections, studies of repair systems are equally as important as studies of "log-kill". Moreover, since in a number of instances drugs behave like radiation (and vice versa) insights gained in drug studies may add to our understanding of radiation effects.

Proposed Course of Project: 1. To get a better insight into the molecular basis of radiation and drug damage, attempts will be made to isolate and analyze nuclear DNA. 2. The mechanism underlying the large survival changes which may be effected in connection with sulfur mustard treatment will be pursued. We hope to test the possibility that these survival changes reflect changes in: DNA bifunctional alkylation; and/or changes in cell wall interactions. 3. In view of the lack of an interaction between nitrogen mustard and X-rays, we will look into the possibility that sulfur mustard and X-rays interact since these agents have other properties in common.

Publication:

Elkind, M. M., Sutton-Gilbert, H., Moses, W. B., and Kamper, C.: Sublethal and lethal radiation damage. Nature 214: 1088-1092, 1967.

Honors and Awards:

The Revulson Lecture, University of Minnesota Medical School, May 1968.

Symposia: 1) The First L. H. Gray Memorial Conference, Northwood England, 1967.

2) The Third Annual San Francisco Cancer Conference, Oct. 1967.

Seminars:

Brookhaven National Lab., Upton, N. Y., Dec. 1967.

Columbia University Medical School, N.Y.C., Jan. 1968.

National Center for Radiological Health, Rockville, Md., Jan. 1968.

Georgetown Univ. Med. School, Washington, D. C., May 1968.

University of Tennessee Med. School, Memphis, April 1968.

University of Virginia Med. School, Charlottsville, May 1968.

University of Minnesota, St. Paul, May 1968.

Serial No. NCI-935
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Synthesis of Nucleic Acids and Protein and Their Relationship to Radiation Effects in Cultured Mammalian Cells

Previous Serial Number: Same

Principal Investigator: Mortimer M. Elkind

Man Years

Total:	2.5
Professional:	2.5
Others:	0

Project Description:

For Objectives, Methods, and Major Findings, see previous NCI-935 report.

Actinomycin D affects lethal and sublethal damage expression. Still, concentrations which are effective in this regard do not completely stop RNA synthesis. Concentrations of puromycin, which have a larger effect on RNA (and DNA) synthesis, do not suppress sublethal damage repair. It appears, therefore, that actinomycin D is effective because of its ability to bind to DNA rather than because of its interference with metabolic processes.

Publications:

Elkind, M. M., Moses, W. B., and Sutton-Gilbert, H.: Radiation response of mammalian cells grown in culture. VI. Protein, DNA, and RNA inhibition during the repair of X-ray damage. Radiation Res. 31: 156-173, 1967.

Elkind, M. M.: Sublethal X-ray damage and its repair in mammalian cells. In Silini, G. (Ed.): Radiation Research. North-Holland Publishing Co., 1967, pp. 558-586.

Serial No. NCI-901
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Response of Skin and Hair to Irradiation

Previous Serial Number: Same

Principal Investigator: H. Rodney Withers

Other Investigator: Mortimer M. Elkind

Man Years

Total:	1.0
Professional:	0.5
Others:	0.5

Project Description:

Objectives: Because of the demands of developing and exploiting a better technique for studying the response of normal tissues to irradiation (see project #902) very little progress was made on this project before it was shelved. New data add nothing qualitatively different from those presented last year.

Publications:

Withers, H. R.: The effect of oxygen and anaesthesia on radiosensitivity in vivo of epithelial cells of mouse skin. Brit. J. Radiol. 40: 335-343, 1967.

Withers, H. R.: Recovery and repopulation in vivo by mouse skin epithelial cells during fractionated irradiation. Radiation Res. 32: 227-239, 1967.

Serial No. NCI-902
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Response of Mouse Intestinal Mucosa to Irradiation

Previous Serial Number: Same

Principal Investigator: H. Rodney Withers

Other Investigator: Mortimer M. Elkind

Man Years

Total:	1.0
Professional:	0.5
Others:	0.5

Project Description:

Objectives:

a. To determine the radiosensitivity of the mucosal epithelial cells of the small intestine.

b. To investigate the role of cellular radiosensitivity in the overall response of intestine to irradiation.

Methods Employed:

a. A segment of intestinal mucosa is exteriorized and irradiated with doses from two opposed X-ray tubes operated at 200 kVp. The numbers of cells surviving per unit area is determined by counting the numbers of colonies of regenerating epithelium 13 days post-irradiation. When the response to two doses of X-rays is to be investigated, the first dose is delivered to the whole abdomen without exteriorizing an intestinal segment.

b. Histological sections prepared from mouse jejunum 3 to 5 days after irradiation are examined and the numbers of micro colonies of regenerating epithelium counted. This permits evaluation of the effects of lower doses of X-rays than is possible with method a.

c. The LD_{50} for radiation-induced "gut-death" is determined for single and divided doses of X-rays.

Major Findings: The epithelial cells lining the crypts of the mucous membrane of the small intestine exhibit X-ray survival characteristics similar

to those of most other mammalian cells thus far investigated except that they have a greater capacity for repair of sublethal injury. This is shown in a comparison of survival from irradiation given as a single dose with that resulting from the same total dose given as two fractions separated in time. It was found that survival increased nearly forty-fold if a dose of 2200R was divided into two fractions (700R + 1500R) separated by an interval of four hours. Recovery after the first four hours proceeds more slowly, and is followed by proliferation of surviving cells. By 24 hours the recovery factor is more than 60 and by four days after a dose of 700R, is about 2000. These findings indicate a very rapid rate for both repair of sublethal injury and cell proliferation.

If the whole abdomen of the mice used in these experiments is exposed to a dose of 1130R, an average of 50% will die from intestinal injury. It may be calculated from the dose-survival relationship determined using the colony counting technique, that more than 99.9% of the mucosal stem cells are sterilized by this dose. A good correlation was found between animal survival from "gut-death" and cellular survival when the patterns of recovery between two doses were compared.

Significance to Bio-medical Research and the Program of the Institute: The results of our experiments emphasize the importance of the repair capacity of intestinal cells in determining the reactions of intestine to irradiation. From a practical viewpoint, this means that X-ray therapy of abdominal tumors, which is frequently limited by incidental intestinal injury, depends for its success on limiting the size of individual dose fractions. It also serves as a warning that special care is needed when new radiation techniques that interfere with intracellular repair of sublethal injury are applied to situations in which intestine is also irradiated.

Proposed Course of Project: The principal investigator's appointment terminates July 5, 1968.

Honors and Awards:

Seminar: "Dose Response of Jejunal Crypt Cells", M. D. Anderson Hospital and Tumor Institute, October 1967, Houston, Texas.

Address: "Dose Survival Characteristics of Epithelial Cells of the Mouse Small Intestine" at the Annual Meeting of the Radiological Society of North America, December 1, 1967, Chicago, Illinois.

Seminar: "Dose Response Relationships for Mouse Small Intestine", Argonne National Laboratory, February 1968, Chicago, Illinois.

Address: "The Response of Mouse Small Intestine to Two-Dose Irradiation" at the Annual Meeting of the Radiation Research Society, April 1968, Houston, Texas.

Serial No. NCI-936
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: 1. Kinetics of Damage and Recovery in the Hemopoietic System.
2. Effects of Chemical Agents on the Response of Tumor-bearing mice to Irradiation.

Previous Serial Number: Same

Principal Investigator: Willie W. Smith

Other Investigators: Sallie S. Fred, NCI Postdoctoral Fellow, No. 5-F2-CA-34, 81802; Philip Caper, Research Associate; Mrs. Shirley Wilson.

Cooperating Units: Medicine Branch, NCI, Clinical Associates (part time): Drs. Joseph Glass, Joseph Newman, Edward Cahn and Stephan Carter (Project 2).

Man Years

Total:	6.5
Professional:	4.0
Others:	2.5

Project Description:

Objectives and Methods Employed: Ability of the hemopoietic stem cell population to recover from damage by radiation or chemical agents is frequently the limiting factor in cancer therapy. It is our object to learn as much as possible about response to these treatments and processes involved in recovery. Assays are carried out by the method of Till and McCulloch which is based on the ability of stem cells to form colonies in the spleen. Effects of agents used in chemotherapy on the response of tumor-bearing mice to irradiation are also being studied and results analyzed for possible interaction. Vinblastine (VLB) and vincristine (VCR) are two such agents, which also promote early postirradiation hemopoietic recovery.

Major Findings:

Project 1. a) There was a substantial reduction in colony-forming units (CFU) of femur and spleen in mice given VLB. This was followed by recovery and overcorrection, first in CFU, then in nucleated cells. This suggests that a feed-back loop, probably involving a humoral substance, is set in motion by the drop in nucleated cells.

b) The transplantation fraction of CFU was reduced in femoral cells from mice given VLB, endotoxin or irradiation. Colony forming cells in the femur estimated from the transplant fraction were doubled two days after VLB injection, and the concentration increased from three per thousand to two per hundred nucleated cells.

c) Femoral CFU in mice given VLB or VCR two days before irradiation began to increase immediately, without the postirradiation dip or lag phase which always occurs in controls. There was a corresponding advance in recovery of bone marrow cellularity and peripheral blood granulocyte, lymphocyte and hemoglobin concentrations.

Large numbers of microscopic granulocytic colonies were seen in the spleen four to six days after irradiation in mice given endotoxin one day before exposure. These disappeared and a large number of erythroid colonies appeared 8 to 10 days after irradiation. An injection of endotoxin immediately after irradiation had a similar though smaller effect.

Project 2. a) Tolerance to radiation was increased in mice bearing lymphosarcoma P1798 by pretreatment with VLB. Effectiveness of radiation was relatively greater when the tumor cells were reduced either by pretreatment or by giving a smaller inoculum. This increased response to radiation was not seen in the mice bearing L1210. Pretreatment with VLB or VCR did not appear to affect the radiation sensitivity of the tumor.

b) Mice bearing most cell tumor P815 were treated with the following chemical agents prior to irradiation: vincristine, cytoxan, procarbazine, hydroxyurea, cytosine arabinoside and daunomycin. Effects on survival were generally additive with effects of irradiation, no evidence of potentiation being seen. Pretreated mice responded to irradiation like untreated mice that had the same survival time due to a lower inoculum.

c) Toxicity of actinomycin D and radiation given within a few hours was greater than that of either alone. These experiments are being continued, as well as studies of effects of combined treatments with this agent and with 5-diododeoxy uridine, 5-fluorodeoxy uridine and cytosine arabinoside in mice bearing leukemia L1210.

Significance to Bio-medical Research and the Program of the Institute: In cancer therapy, the resulting hemopoietic damage often limits the amount of treatment that can be tolerated. An understanding of the processes involved in this damage and in recovery is of obvious interest. Certain agents effective in chemotherapy were shown also to induce an early post-irradiation hemopoietic recovery. These agents are cytotoxic, and may promote recovery by initiating a feed-back loop that involves a humoral substance. Demonstration of such a substance would be of considerable basic and possibly practical significance.

To express experimental results with CFU in terms of stem cells, knowledge of the transplantation fraction is essential. Of more general importance is an understanding of the cellular processes controlling transplantability to which these experiments contribute.

Analysis of results of combined chemical and radiation therapy in experimental tumors demonstrated effects of 1) promoting early postirradiation hemopoietic recovery and 2) reducing tumor cells to levels at which the relative effectiveness of radiation increased. Increased radiation sensitivity of the tumor has not so far been encountered.

Proposed Course: Studies of transplantation fractions for CFU under conditions of experimental interest are being extended. The fraction for initial transplantation cannot be obtained with the CFU technique, but first, second and third transplantation fractions for marrow cells labeled with tritiated thymidine are being determined. These will be compared with second and third fractions for CFU and inferences made concerning the stability of transplantation fractions.

Problems of stem cell survival and differentiation are being investigated in mice treated with endotoxin before or shortly after irradiation. Studies of the early differentiation and disappearance of numerous granulocytic colonies in the spleen and later differentiation of erythroid colonies will be continued. Mice with a genetic deficiency in erythrocytes and in CFU will also be used in these studies.

If the proliferative recovery of stem cells is initiated by a physiological humoral agent, it is possible that such an agent can be demonstrated in the serum. Attempts will be made to show its presence. At the same time, we will seek evidence that stem cell recovery is related primarily to a host factor or primarily to the direct action of the chemical agent on the stem cell.

Experiments on combined treatments in tumor-bearing mice will be continued. We are particularly interested in obtaining evidence of increased radiation sensitivity of the tumor.

Publications:

Smith, W. W., Wilson, S. M.: Effects of vinblastine and vincristine on survival and hemopoiesis in irradiated mice. J. Nat. Cancer Inst. 39: 1055-1066, 1967.

Smith, W. W., Wilson, S. M. and Fred, S. S.: Kinetics of stem cell depletion and proliferation: Effects of vinblastine and vincristine in normal and irradiated mice. J. Nat. Cancer Inst. 40: (April) 1968.

Fred, S. S. and Smith, W. W.: Radiation sensitivity and proliferative recovery of hemopoietic stem cells in weanling as compared to adult mice. Radiation Res. 32: 314-326, 1967.

Fred, S. S., Wilson, S. M. and Smith, W. W.: Role of intestinal injury in 30-day radiation lethality of weanling mice. In Sullivan, M. F. (Ed.): Gastrointestinal Radiation Injury, Monographs on Nuclear Medicine and Biology, No. 1, Amsterdam, Excerpta Medica Foundation, 1968, pp. 413-420.

Fred, S. S. and Smith, W. W.: Induced changes in transplantability of hemopoietic colony forming cells. Proc. Soc. Exp. Biol. Med. (in press).

Serial No. NCI-938
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Antibody Formation Following Multiple Irradiation and Immunization Procedures

Previous Serial Number: Same

Principal Investigator: Laurence R. Draper

Other Investigator: H. T. Foley (Radiation Branch)

Man Years

Total:	3.0
Professional:	1.5
Others:	1.5

Project Description:

Objectives: To determine the capacity for and mode of natural and specific anti-sheep hemolysin formation in rabbits following single and repeated exposures to ionizing radiation.

Methods Employed: X-rays (200 kvp) were used for single acute 400 to 600 R whole body exposures. A Co^{60} teletherapy unit was used for chronic exposures. Serum natural hemolysin was assayed by colorimetric and micro-titration methods. Antibody avidity was measured in terms of inter-erythrocyte transfer. Transplantation immunity was tested by ear skin graft techniques. Various physico-chemical procedures were used to characterize molecular species of antibody in antisera. Immunosuppressant agents and extirpation procedures were used to modify and study recovery phenomena after irradiation.

Major Findings: Natural antisheep hemolysin arises in rabbits through minimal stimulation by ubiquitous Forssman and Forssman-like antigens. Antibody avidity studies indicate that the specificity of the pertinent antigen varies with time.

Acute Irradiation interrupts the induction of natural hemolysin production but in the continued presence of antigen, recovery is accompanied by a return of natural antibody production which leads to enhanced serum antibody titers.

6-mercaptopurine and cyclophosphamide, immunosuppressive drugs, given in various regimens do not impair natural antibody production in non-irradiated animals. Cyclophosphamide when administered in 10 consecutive days at a rate of 25 mg/kg/day intraperitoneally will modify the timing and extent of the resumption of natural antibody synthesis after acute exposure to 500 R, provided the drug regimen is begun before the start of antibody titer recovery.

Passively transferred specific anti-sheep hemolysin was ineffective in altering the level of natural antibodies in normal rabbits or in rabbits X-irradiated with 500 R.

Prolonged irradiation also depresses natural antibody titer in unimmunized rabbits. The irradiation protocol consisted of daily 25 R whole body exposures over a period of 7 to 8 months. As the total dose exceeded 1000 R natural hemolysin titer declined to about 50% of the control value. With increasing accumulated exposure mean titer declined progressively to less than 1% normal levels (5500 R). In some surviving rabbits titer remained below detectable levels for 1 to 3 months.

Several animals which had survived a total exposure of 7000 R showed an impaired capacity to respond to a specific challenge with sheep erythrocytes.

The peripheral lymphocyte population in the chronically irradiated rabbits decreased to about 50% normal after 250 to 500 R and remained at that level for the remainder of the irradiation course. Peripheral granulocytes and hemoglobin were only minimally decreased. The irradiation significantly hampered the growth of the rabbits based on body weight gain throughout the course of the experiment.

While humoral natural antibody production was severely depressed, the ability of the irradiated rabbits to reject skin homografts was virtually normal.

Immediately after the last exposure, the surviving animals were sacrificed and various tissues were excised for histological studies. While the spleen, femoral bone marrow and mesenteric lymph nodes showed moderate to severe cellular depletion, the appendix was markedly disorganized and depleted of cells. The correlation between appendical destruction and the depression of hemolysin titer was striking. In contrast, the thymuses of the irradiated animals showed only minor abnormalities. Lung and liver were morphologically normal.

In ancillary study in collaboration with Dr. Robert Kagan (Radiation Branch, NCI) sera from several hundred patients undergoing radiotherapy are being screened for natural heterophile antibody to determine whether antibody titers are related to disease states and therapeutic treatments.

Significance to Bio-medical Research and the Program of the Institute: These studies have particular relevance to the effects of radiation exposure on immunological capacity. Natural antibody formation in rabbits provides a unique system for continuous appraisal of humoral responsiveness in individual animals. It also permits the study of the recovery of immune competence after irradiation without imposing abnormal antigenic stimulation.

Proposed Course of the Project: These studies will be terminated at this Institute in August 1968. However, in light of the results mentioned above, experiments are already in progress concerning the roles of the rabbit appendix and other gut-associated lymphoid tissues in the production of natural hemolysin and in the response to a specific immunization with sheep erythrocytes. To this end, experiments involving extirpation, of the appendix, sacculus rotundus and Peyers patches have begun. In addition, appendiceal tissue is being assayed for individual antibody producing cells by plaque techniques to characterize the relative immunological contribution of these tissues.

Publication:

Draper, L. R.: Suppression of natural hemolysin titer in rabbits exposed daily to gamma radiation. Nature 215: 848-849, 1967.

Serial No. NCI-946 (New)
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Qualitative Changes Induced in Antibody by Irradiation

Previous Serial Number: None

Principal Investigator: Laurence R. Draper

Man Years

Total:	1.5
Professional:	1
Others:	0.5

Project Description:

Objectives: IgG and IgM antibody is inactivated by heavy irradiation. The dose response curves describing this inactivation of hemolytic antibody indicate that IgG loses activity upon the inactivation of a single target, whereas, IgM antibody may suffer more than one "hit" without losing its ability to bind to the target red cell and participate in the production of a lytic event. Studies have been initiated to determine whether radiation will also modify the binding strength (avidity) of either of the two types of antibodies and whether the sites determining hemolytic activity and avidity are the same.

Methods Employed: Dried samples of a variety of antibody preparations containing IgG, IgM or mixture of the two are irradiated at dry ice temperatures with 2.5 MeV electrons. The samples are reconstituted and titrated for hemolytic activity and characterized in terms of avidity by means of intracellular transfer methods.

Major Findings: Although these studies are in their preliminary stages, the results to date indicate that IgG hemolysin which retains hemolytic activity after irradiation shows no change in affinity for its target antigen. On the other hand, irradiation of IgM hemolysin may produce a population of "damaged" antibody molecules which retain their ability to initiate a lytic event but show a markedly reduced binding affinity for the target molecule.

Significance to Bio-medical Research and the Program of the Institute: These studies have particular relevance to the knowledge of the action of ionizing radiation on the biological properties of macromolecules and hopefully will add to the understanding of the relationship between the structure and function of those molecules.

Proposed Course of the Project: Additional data will be collected on a greater variety of immune sera and fractions thereof. Sub units of the various antibody types will also be used to investigate more closely the region on the antibody molecule involved in avidity. This project will be terminated at this Institute in August 1968.

Publications: None

Serial No. NCI-941
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunogenetic factors affecting transplantation of hematopoietic tissue in irradiated mice: Modification of functional capacity of bone marrow cells

Previous Serial Number: Same

Principal Investigator: Delta E. Uphoff

Man Years

Total:	2-1/3
Professional:	1
Others:	1-1/3

Project Description:

Objective: To study factors altering the functional capacity of immunologically competent bone marrow cells with the ultimate goal of increasing the feasibility of transplanting bone marrow and other organs or tissues.

Methods Employed: Lethally irradiated inbred mice or F_1 hybrids were recipients for marrow transplants from normal or pretreated syngeneic (same strain), allogeneic (foreign strain), or F_1 hybrid donors. An *in vitro* incubation method was developed which facilitated this study. Marrow preparations were incubated *in vitro* with cellular antigens, or soluble and particulate noncellular antigens for various intervals and at various temperatures before inoculation into lethally irradiated recipients. Survival patterns of recipients varied with conditions of *in vitro* incubation. Survivors were tested with skin homografts to demonstrate tolerance or chimerism (a successful permanent marrow allograft). Genetically high leukemic strains were studied for altered incidence of neoplasms. Humoral antibody (agglutinin and hemolysin) tests were conducted in collaboration with L. R. Draper (NCI-938).

Major Findings:

A. Marrow transplantation in irradiated mice:

1. Time and temperature of *in vitro* contact of marrow cells with washed allogeneic erythrocytes (rbc) affected the protective capacity of inoculated marrow. Strains differed in the rate with which preincubated marrow regained its ability to prevent hematopoietic depletion in lethally

irradiated syngeneic mice. "Fast reacting" strains regained their protective capacity following an incubation of 35 minutes at 25-30°C. "Slow reacting" strains had a reduced survival under these conditions but regained their protective capacity when the marrow was preincubated 45-60 minutes. Reducing the temperature to 23°C appeared to retard the interaction between the marrow and the antigen during incubation. Reasons for this critical period during which preincubated marrow was incapable of sparing the life of the irradiated recipient were unknown. The observed early mortality appeared analogous with the long recognized fact that survival depended upon the genetic relationship between host and donor and that allogeneic marrow failed to protect all strains with equal facility.

2. The most complete immunogenetic investigation of effects of in vitro incubation were conducted using high leukemic AKR mice, low leukemic CBA mice, and reciprocal hybrids, (CBA × AKF)F_1 and (AKR × CBA)F_1.

Incubation of "fast reacting" AKR marrow with rbc from CBA, (CBA × AKR)F_1 and (AKR × CBA)F_1 mice resulted in good protection of lethally irradiated AKR recipients. Incubation with hybrid rbc resulted in good long-term survival and development of specific skin allograft tolerance toward CBA donors but they rejected indifferent skin allografts from C_{58} donors even though all 3 strains were of the same histocompatibility-2 phenotype ($H\text{-}2^k$). AKR mice inoculated with AKR marrow preincubated with CBA rbc developed a typical secondary disease syndrome that was lethal to 25% of the mice. This syndrome could be a graft-versus-host reaction only by assuming that the preincubation procedure interfered with the "self-recognition" mechanism. Supporting this hypothesis was the fact that surviving mice were specifically tolerant to CBA skin allografts. Allogeneic CBA marrow preincubated with the 3 types of erythrocytes resulted in good initial protection but exhibited an accelerated graft-versus-host reaction. Late mortality was increased from 7% with normal CBA marrow to 38% with CBA marrow preincubated with AKR or (AKR × CBA)F_1rbc and 67% following incubation with (CBA × AKR)F_1rbc. "Tolerance" resulted from in vitro incubation of syngeneic marrow with allogeneic erythrocytes but "sensitization" resulted from incubating allogeneic CBA marrow with host tissue antigens. The "degree" of sensitization was apparently independent of amount of antigen since exposure to both AKR and (AKR × CBA)F_1rbc produced identical results. A more severe G-v-H reaction with an earlier onset of mortality occurred when the marrow donor and the female component of the hybrid cross were the same strain i.e., CBA marrow incubated with (CBA × AKR)F_1rbc.

Survival patterns differed when "slow reacting" CBA marrow was preincubated with allogeneic erythrocytes and inoculated into CBA recipients. An in vitro incubation of 35 minutes with either AKR or hybrid rbc greatly reduced the 14-day survival and a severe secondary disease syndrome was lethal to a majority of the protected mice. The few mice that recovered were tolerant to AKR skin allografts. These identical marrow preparations that failed to protect the syngeneic recipient, protected the allogeneic AKR recipients indicating that the preincubated marrow cells were fully

viable. Incubation of allogeneic AKR marrow with CBA or hybrid rbc resulted in a late mortality pattern similar to that described for the AKR recipient. Normal allogeneic AKR marrow produced a G-v-H reaction lethal to 40% of the CBA recipients; incubation of AKR marrow with CBA or (CBA × AKR)F_1rbc resulted in a G-v-H mortality of 68 and 64% respectively while incubation with (AKR × CBA)F_1 increased late mortality to 94%. In both cases, the greatest degree of "sensitization" occurred when the allogeneic marrow donor and the female component of the hybrid cross represented the same strains.

The crucial demonstration that this was an immunogenetic phenomenon was that incubation of marrow from either hybrid with rbc from either parental strain did not alter the protective capacity of the marrow nor the long term survival of the host.

In addition to inducing "tolerance" in mice receiving preincubated syngeneic marrow and "sensitizing" allogeneic marrow to host tissue antigens the incidence of thymic lymphomas in the high leukemic AKR recipients was altered by these preincubation procedures. Although only about 50% of the mice in each group have died the range in percentage of AKR mice dying with thymic neoplasms was 8% to 67% depending upon the pretreatment of the marrow. Lethally irradiated AKR mice inoculated with normal AKR or CBA marrow have an incidence of thymic neoplasms of 67 and 50% respectively while inoculation of normal hybrid marrow resulted in 35 to 50% of the mice dying of thymic neoplasms. Incubation of CBA marrow with the 3 types of rbc reduced the thymic neoplasms to one in each of the 3 groups or a combined incidence of 9% in contrast to 50% for nonincubated CBA marrow. Inoculation of syngeneic AKR marrow preincubated with CBA rbc or rbc from (CBA × AKR)F_1 hybrids had an incidence of thymic neoplasms of 8 and 20% respectively. Inoculation of AKR marrow with (AKR × CBA)F_1rbc did not reduce the incidence of thymic neoplasms which was 64% for preincubated marrow and 67% for nonincubated AKR marrow. With less than 50% of the mice in each group dead, it appeared that inoculation of preincubated hybrid marrow was not effective in reducing the incidence of these neoplasms which had already reached 30%.

The occurrence of non-thymic leukemias was limited to single cases in any given group so far. There were 2 striking observations on these mice. Non-neoplastic mortality resulted from severe kidney damage and an extremely small remnant of thymic tissue was usually present. It was postulated that the reduced incidence of thymic neoplasms resulted from an immunogenetic distraction of the thymus. It was not apparent why incubation of AKR marrow with (AKR × CBA)F_1rbc failed to produce any reduction in the incidence of neoplasms while (CBA × AKR)F_1rbc was reasonably effective. It will be of interest to know whether a similar difference will be apparent in the hybrid recipients.

The AKR mice used in the radiation experiments were 3 months of age at the time of treatment. A similar group of 3-month-old AKR females were inoculated with AKR marrow preincubated with CBA rbc but without

exposure to irradiation. The thymic neoplasms developed at approximately the same age as in the noninoculated nonirradiated female AKR mice presumably because of a lack of growth stimulus for the marrow graft. It will be of interest to know whether this phenomenon requires exposure to lethal irradiation or whether a high sublethal exposure would also be effective.

Retired breeders were the only available AKR mice that exceeded 3 months of age. When mice 7 to 10 months of age were irradiated and inoculated with AKR marrow preincubated with CBA rbc there was a reduced incidence of thymic lymphomas similar to that observed for the mice treated at 3 months of age. The "malignant transformation" that resulted in death of untreated mice was either an acute, rapidly progressing process of short duration, or it was a reversible process since 90% of the AKR mice that survive longer than 6 months die of leukemia.

3. The absence of demonstrable humoral antibody in 90% of the lethally irradiated mice inoculated with syngeneic marrow preincubated with soluble or particulate antigens was consistent with the finding for tissue antigens that marrow syngeneic to the recipient was rendered tolerant to the antigen used in the preincubation procedure.

B. Maternal effects: a determination of differences between parental, foster nursed on ova transfer substrains:

1. It was apparent from previous experiments that foster nursing affected radiation sensitivity. To test this hypothesis, the radiosensitive BALB/c mice were foster nursed on radioresistant CBA foster mothers then subsequently inbred. Exposure of parental strain BALB/c to 450R at 3 months of age resulted in a mortality of 52% within 30 days while exposure of the foster nursed, substrain BALB/cfCBA to 450R resulted in only 21% mortality. These results indicate that the increased radioresistance of germ-free and specific pathogen-free animals now attributed to their disease-free state may totally or partially result from the foster nursing process.

C. Induction of immunological tolerance:

1. Inoculation of marrow rendered tolerant by means other than *in vitro* preincubation resulted in an altered survival pattern similar to that observed after inoculation of preincubated marrow. A hypothesis was formulated to explain these results. The "tolerant" marrow no longer recognized the allogeneic strain to which it was rendered tolerant as "foreign" but does recognize syngeneic tissue as "foreign" or fails to recognize "self." The parallel that appears to exist between the results of these two types of experimental procedures was not absolute since preincubation of marrow altered the incidence of thymic lymphomas in AKR mice but neonatally induced tolerance in AKR mice had no such beneficial effect.

Significance to Bio-Medical Research and the Program of the Institute: This in vitro preincubation method offered a new approach to investigation of "tolerance", "sensitization" and other aspects of the immune response. This test system was sensitive and specific for certain types of cell modifications. It clearly demonstrated a nongenetic difference in the erythrocytes of reciprocal hybrid. These observations made apparent the lack of information pertaining to the genetics of the cell membrane. With the increased interest in immunotherapy of tumors, more information must be made available on methods by which the antigenicity of cells may be increased. This is particularly critical since the same methods that produced an increased graft-versus-host reaction produced "tolerance" under other genetic conditions.

Proposed Course of Project: An investigation has been started to determine the changes that occur in AKR mice treated with AKR marrow preincubated with (CBA × AKR)F_1rbc that result in a secondary degeneration of the thymus and a reduced incidence of thymic lymphomas as compared with mice receiving AKR marrow preincubated with (AKR × CBA)F_1rbc which does not reduce the number of thymic neoplasms. Other aspects of this project reported in previous years are being continued.

Publications:

Uphoff, D. E.: Skin allograft "tolerance" induced in adult mice by by transplantation of syngeneic marrow incubated in vitro with allogeneic erythrocytes. In Advances in Transplantation, Proceedings of the First International Congress of the Transplantation Society, Paris 1967 (Dausset, J., Hamburger, J., and Mathé, G., eds.). Copenhagen, Munksgaard, 1968, pp. 469-473.

Serial No. NCI-942
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effects of Ionizing Radiation on Macromolecules of Biological Importance.

Previous Serial Number: Same

Principal Investigator: Peter Riesz

Other Investigators: F. H. White, Jr. (Cellular Physiology Section, Laboratory of Biochemistry, National Heart Institute).

Man Years

Total:	2.5
Professional:	1.0
Others:	1.5

Project Description:

Objectives: In recent years the primary aim of this project has been to investigate the molecular damage produced in biologically active macromolecules by ionizing radiation and to determine what part of the molecular damage is responsible for the loss of biological activity. This work has been carried out with macromolecules in the dry state where the energy is absorbed directly by the macromolecule and diffusion is limited. In dilute aqueous solutions the effects on biologically important macromolecules are due entirely to the reactions of freely diffusible radicals formed by the radiolysis of water. Both of these approaches are required to understand the more complex situation when living cells are irradiated, since the damage done to the macromolecule in the cell is the sum of the damage done directly to the molecule, as measured by radiation effects in the dry state, and the damage produced by the reactions of water radicals as determined by experiments in aqueous solutions.

The long-lived free radicals produced by gamma-radiolysis of dry proteins have been studied by previous investigators using the technique of electron spin resonance (ESR). It was shown that, when proteins are irradiated at liquid nitrogen temperature, ESR centers are formed ("primary" radicals) which on heating to room temperature are irreversibly changed to give spectra which are identical with those found after radiolysis at room temperature ("secondary" radicals).

In the course of work at NIH a new method for identifying the "secondary" free radicals in gamma-irradiated macromolecules has been developed [Riesz, White and Kon, J. Am. Chem. Soc. 88, 872-877 (1966)]. This consists of exposing the trapped radicals, produced by the gamma-radiolysis of proteins in vacuo, to a tritiated radical interceptor which reacts with all of the radicals to give stable carbon-tritium bonds. Subsequent hydrolysis, amino acid analysis and tritium counting allow the determination of the tritium distribution among the amino acids, which appears to be a measure of the "secondary" free radical distribution.

Very recently, it has been found possible to utilize the same approach for determining free radical distributions at -79°C. Since the ESR spectra at -196°C and at -78°C are essentially identical, it now appears possible to obtain a more detailed understanding of processes occurring much earlier than the formation of the "secondary" radicals.

In addition to the study of free radicals produced by ionizing radiation, by ultra violet or by H atom bombardment, the radical interceptor technique can be exploited to prepare tritiated proteins and to study the conformation of macromolecules in the lyophilized state. The possibility also exists for the development of methods for comparing the conformation of macromolecules in aqueous solutions with the three-dimensional structure in the crystalline state, where it is known. If such experiments are carried out with enzymes in the presence of substrate or inhibitor, or with antibodies complexed with antigen, surface labelling near the active site will be reduced so that information about the location of the active site may be obtained.

Methods Employed: When dry proteins or nucleic acids are irradiated with gamma rays or ultraviolet radiation in the absence of air (at 10^{-4} mm pressure), trapped free radicals are generated. Subsequent exposure to tritiated hydrogen sulfide or hydrogen iodide leads to the formation of carbon-tritium bonds. After removal of exchangeable tritium, the protein is hydrolyzed, the amino acids are separated on an automatic amino acid analyser and the tritium content of each amino acid is determined by means of an associated flow counter. It has been shown that the tritium distribution so obtained corresponds closely to the free radical distribution at the time of exposure to the tritiated free radical interceptor.

The kinetics of the reaction of free radicals in the irradiated macromolecule with various radical interceptors were followed by means of electron spin resonance spectroscopy in collaboration with Dr. Hideo Kon (Section on Molecular Biophysics, LPB, NIAMD).

Major Findings:

I. The Effect of Temperature and Conformation on the Free Radical Distributions of Several Gamma-Irradiated Dry Proteins.

A. The distributions of free radicals on carbon in some gamma-irradiated lyophilized native proteins (ribonuclease, chymotrypsinogen, lysozyme

and nagarse) and in denatured proteins (carboxymethylated reduced ribonuclease chymotrypsinogen, lysozyme and urea reoxidized ribonuclease) have been determined at -79°C and 25°C.

B. At 25°C each native protein has a characteristic tritium distribution but the reduced carboxymethylated (denatured) proteins have much more nearly the same distribution. The urea reoxidized ribonuclease with a random conformation but intact disulfide bridges has the same distribution as the carboxymethylated reduced ribonuclease, a protein with a random conformation and broken disulfide bridges.

C. At -79°C, the tritium distribution as measured by normalized specific activities, is approximately the same for all of the native and denatured proteins examined. At this temperature the probability of a free radical being located on a particular amino acid residue is independent of primary sequence, chain length and conformation. The distribution at -79°C is approximately the same as that obtained for the denatured proteins at 25°C.

D. The mechanism of formation of the free radical distribution in native proteins at 25°C can be divided into two groups of processes. The first group takes place below -79°C and gives similar distributions for all proteins so far examined. The second group, which occurs above -79°C and is influenced by the specific conformation of each native protein. A general tendency of this second group of processes is to reduce the specific activities of the non-polar residues which are moved to the interior of the native protein when the random polypeptide chain is folded to give the biologically active protein.

E. Tritium distributions obtained by "beta-labelling" (tritium incorporated as a result of beta-decay in the presence of tritiated hydrogen sulfide) and by "gamma-labelling" (tritium incorporated as a result of radical formation by gamma-radiolysis) are identical at -79°C but different at 25°C. The effect of temperature on "beta-labelling" has been studied from -79°C to 70°C. "Beta-labelling" at room temperature involves two separate mechanisms; one of these is equivalent to gamma labelling, the radicals being formed by the absorption of beta particles from that portion of the tritium which is located on the exchangeable sites (i.e., attached to oxygen or nitrogen) of the protein molecule. Consistent with this conclusion, it was found that the effect of added ammonia gas on the gamma-labelled and beta-labelled tritium distribution was the same. The second mechanism has not been investigated so far; a likely possibility is the contribution of processes resulting from self-radiolysis in the gas phase.

F. The major conclusion that the tritium distribution for native proteins at -79°C is approximately equal to the tritium distribution of denatured proteins at 25°C can be understood by considering the effect of conformation on the intra- and intermolecular radical migration processes which occur on warming the protein from -79°C to 25°C.

II. The Stereochemistry of Tritium Labelling by the Radical Interceptor Technique. (with F. H. White, Jr.)

From studies of the ESR spectra of gamma-irradiated proteins it has been suggested that the carbon radicals are probably located on the alpha carbons of the polypeptide chain. If this should be the case, the possibility arises that such alpha-radicals may racemize prior to reaction with tritiated hydrogen sulfide leading to tritiated d-amino acids. Carbon free-radicals generally have planar configurations, although it is difficult to be certain whether in a dry polypeptide such planar configuration will be readily permitted.

Hydrolysates of "gamma-" and "beta-labelled" ribonuclease were treated with d-amino acid oxidase under conditions which destroy 100% d-methionine, 83% d-proline, 76% d-isoleucine, 40% d-phenylalanine, 29% d-valine and 23% d-alanine. The specific activities of these amino acids were not changed (within experimental error) by treatment with d-amino oxidase. The absence of tritiated d-amino acids for the strongly labelled proline and methionine and the weakly labelled other amino acids (alanine, isoleucine, valine, phenylalanine) is consistent with either of two hypothesis:

1. Free radicals on these amino acids are not located on the alpha carbon.

2. Free radicals are located on the alpha carbon but such radicals or lyophilized proteins are not able to assume a planar configuration.

Further studies to locate the position of the tritium atoms in these amino acids are planned. The absence of tritiated d-amino acids is of practical interest in connection with the preparation of tritiated proteins which appear to show little or no structural alteration.

III. The Free-Radical Interceptor Technique as a Means for the Preparation of Tritiated Macromolecules. (with F. H. White, Jr.)

Chromatography of tritiated ribonuclease labelled by the present technique has shown that 85% of the radioactivity elutes with the native peak during ion-exchange chromatography. A similar result has been found for tritiated lysozyme. Further, repeated crystallization did not remove any carbon-bound tritium from tritiated lysozyme. These results suggest that little or no structural alteration occurred during the tritiation procedure and that therefore this method may prove useful in the preparation of tritiated proteins which could be employed in a wide variety of biological reactions. The present method has certain advantages over the Wilzbach labelling technique, which involves the use of pure T_2 gas as the labelling reagent, since only of the order of one-hundredth as much gaseous radioactivity is involved. Much work remains to be done to explore the possibility of heterogeneity due to subtle structural damage.

IV. A Chain Reaction of H_2S with Free Radicals in Irradiated Dry Collagen and in Mixtures of Ribonuclease with Polyproline.

The collagen molecule consists of three helical coils twisted to a

triple helix with dimensions of about 3000 × 15 Å. The molecular weight of each of the three alpha chains is approximately 95,000. Highly purified samples of native rat skin collagen were obtained from Dr. K. A. Piez of the Laboratory of Biochemistry, NIDR. Partially denatured collagen was prepared by dissolving the native collagen in water at 55°C. G values for radical formation in native collagen are about 3-4 radicals/100 ev., of the same order of magnitude as for globular proteins. It was therefore surprising to find that tritium incorporation for both "gamma-" and "beta-labelling" of native and denatured collagen was about 10-30 times greater than for the globular proteins studied previously. Preliminary experiments indicate that this different behavior is due to the high content of proline (12.3% of residues) and of hydroxyproline (9.4%), rather than to the unique structural features of the collagen or the high glycine content (33%). 50:50 mixtures by wt. of ribonuclease with polyproline, polyhydroxyproline and polyalanine have been examined.

For the case of polyhydroxyproline and polyproline, but not for polyalanine, the specific activity of ribonuclease was about five times greater than in the absence of a second component. In addition, the presence of polyproline and of polyhydroxyproline caused large changes in the tritium distribution among the polar amino acids of ribonuclease. Further studies of the mechanism responsible for these unexpected observations are in progress.

V. Conformational Studies in the Lyophilized State.

It is difficult to obtain evidence regarding the conformation of proteins in the lyophilized state. Tritiated radical scavengers may be used to distinguish between surface and interior residues in lyophilized macromolecules in the following manner. The gamma-irradiated lyophilized protein is first treated with an unlabelled radical scavenger whose molecules are so bulky that they cannot react with radicals on theinterior residues and will donate H atoms only to surface residues. Subsequently, this radical scavenger is removed and tritiated hydrogen sulfide is added to label the interior residues with tritium.

Preliminary experiments were undertaken in which gamma-irradiated ribonuclease is first exposed to unlabelled isoamylmercaptan at 15 mm pressure for three hours and then to tritiated hydrogen sulfide for four hours. The most striking difference from the tritium distribution of the "standard" labelling experiment (i.e., one in which only tritiated hydrogen sulfide is used) is the decrease in the specific activity of lysine. The percentage of the total tritium in non-polar residues increases from 25 to 39, that of polar residues decreases from 75 to 61. Assuming that the radical distribution in molecules on the surface and in the interior of the solid particles are the same, these results indicate that most of the lysines are surface residues in the lyophilized state.

VI. Conformation of Proteins in Aqueous Solution.

It is of interest to compare the macromolecular conformation of globular

proteins in solution with the known structure in the crystalline state. Radiolysis of deaerated aqueous protein solutions leads to the formation of carbon radicals on the protein. In the presence of a reactive T-donor, it may be possible to favor the reaction of the protein radicals tc abstract tritium from the T-donor and suppress the reaction with other radicals in the system. Moreover, if the T-donor is of sufficient size, only the surface protein radicals will be labelled.

Attempts to carry out such experiments with tritiated isopropylcyanide and tritiated chloroform in aqueous lysozyme solutions saturated with nitrous oxide lead to the introduction of microcuries/mg amounts of non-exchangeable tritium into the protein. However, after acid hydrolysis, amino acid analysis and counting, only a negligible protion of tritium was associated with the normal amino acids. Further studies involving the same general approach should be undertaken.

Significance to Bio-medical Research and the Program of the Institute: The development of improved methods of radiation therapy requires an understanding of the effects of ionizing radiation on macromolecules of biological importance.

Proposed Course of Project:

A. To quantitatively compare total tritium incorporation with radical concentrations determined by ESR, the tritium isotope effect in the labelling reaction must be known. This can be calculated from results of experiments in which the total tritium incorporation with tritiated hydrogen sulfide and with tritiated deuterium sulfide are compared.

B. In the dry state, comparison of free radical distributions in proteins, nucleoproteins and bacterial viruses when these are exposed to gamma rays, ultra violet and hydrogen atoms generated by microwave discharge.

C. In the dry state, at -79°C and 25°C, to determine the distribution of tritium within the residues of a given amino acid and to locate the carbon atoms to which tritium is attached in a given residue.

D. Investigation of the chromatographic homogeneity of tritiated proteins and antibodies prepared by the radical interceptor technique.

E. Development of methods for comparing the conformation of proteins in the lyophilized state and in aqueous solutions with the known three-dimensional structure in the crystalline state.

F. Development of a general method for studying the active site of enzyme-substrate, antibody-antigen and in mRNA-ribosome complexes.

Publications:

Ormerod, M. G. and Riesz, P.: An unusual reaction of hydrogen sulfide and hydrogen iodide with free radicals in irradiated dry deoxyribonucleic acid. Biochim. Biophys. Acta 149: 451, 1967.

Riesz, P. and White, F. H., Jr.: Determination of free radicals in gamma irradiated proteins. Nature 216: 1208, 1967.

White, F. H., Jr. and Riesz, P.: The free-radical interceptor technique as a means for the preparation of tritiated proteins. Biochem. Biophys. Res. Comm. 30: 303, 1968.

Riesz, P. and White, F. H., Jr.: Tritiated free-radical scavengers in the study of the irradiated protein molecule. In Gould, Robert F. (Ed.): Advances in Chemistry. Washington, D. C., American Chemical Society, in press.

Serial No. NCI-943
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Effect of Ionizing Radiation Upon Information Transfer

Previous Serial Number : SAME

Principal Investigator: Charles R. Maxwell

Other Investigator: Elizabeth S. Maxwell,
Laboratory of Molecular Biology, NIAMD

Man Years

Total:	2
Professional:	1
Others:	1

Project Description:

Objectives: The specific objective of this investigation has been to study the effect of ionizing radiation upon ribosomes isolated from the rat liver with respect to their physical state and their ability to synthesize protein in a cell free system.

For General Objectives and Methods Employed see previous reports.

Major Findings: We have previously reported that Co^{60} γ-irradiation of isolated liver ribosomes before they were used as a part of a cell-free protein synthesizing system: 1) reduced the ability of the ribosomes to synthesize both native protein with attached endogenous messenger and artificial protein with added synthetic RNA, 2) degraded the larger polysomes to smaller polysomes and some damaged nucleotides, 3) enhanced the ability of the ribosomes to bind artificial messenger, 4) decreased the small ribonuclease activity normally associated with a ribosomal preparation and 5) decreased the relative rate at which a protein synthesizing system approaches the final maximum yield of protein. We can now add 1) that the irradiated ribosome apparently retains its ability to recognize the genetic code and 2) that irradiation does not produce dead (inert or stable) polysomal fragments.

The incorporations of C^{14}-labelled proline, valine, alanine, isoleucine, threonine, tryptophane or glutamic acid into acid insoluble proteins by a protein synthesizing system using irradiated ribosomes were not enhanced by the addition of poly U (which directs synthesis of polyphenylalanine) and C^{12}-phenylalanine. Control experiments with C^{14}-phenylalanine showed that the system was synthesizing poly-phenylalanine

but it was not miss-reading and including any of the other amino acids tested. We conclude that the irradiated ribosome retains the ability to recognize the "code."

Sucrose density profiles of irradiated and unirradiated ribosomes have shown that irradiation degrades the larger polysomes to smaller polysomal fragments. This is accompanied by a marked decrease in the ability of the irradiated ribosomes to synthesize "acid insoluble protein." The possibility that this decrease in synthesis was caused by the inability of the fragments to respond to the synthesizing system has been investigated. Sucrose density profiles of irradiated and unirradiated ribosomes have been observed as a function of time in a complete synthesizing system and in a system lacking only the ATP generating system. In both complete systems all the polysomes were eventually degraded to monomers and dimers. The degradation of the irradiated ribosomes was noticeably slower but then so was the cessation of protein synthesis. There were small but relatively insignificant degradation in both sets of ribosomes in the ATP deficient system. We conclude that radiation does not produce "dead" inert polysomal fragments. These fragments are apparently degraded by some mechanism (probably protein synthesis) requiring the complete synthesizing system. Although we have not done so, it would be interesting to examine the system for small soluble polypeptides.

Significance to Bio-medical Research and the Program of the Institute: Knowledge of the effect of radiation upon the genetic and information transfer mechanism is of great interest to bio-medical research and particularly to cancer, where the cancerous cell may simply be a cell with a faulty information transfer or storage system.

Proposed Course of Project: This project has been terminated and a manuscript is in preparation.

Serial No. NCI-940
1. Laboratory of Physiology
2. Radiation Biology Section
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Production and Nature of Antibody Produced Against Different Physical Forms of the Same Antigen.

Previous Serial Number: Same

Principal Investigator: Laurence R. Draper

Other Investigator: A. A. Hirata (Abbott Laboratories, North Chicago, Illinois)

Man Years

Total:	0
Professional:	0
Others:	0

Project Description:

No further studies were carried out on this project during FY 1967.

The experimental results as described in previous annual report were prepared, submitted and accepted for publication.

Publication:

Draper, L. R., and Hirata, A. A.: Antibody responses in rabbits to soluble and particulate forms of bovine serum albumin. Immunology 15: 23-30, 1968.

ANNUAL REPORT

THE ENDOCRINE EVALUATION BRANCH AND
BREAST CANCER TASK FORCE PROGRAM
July 1, 1967 - June 30, 1968

INTRODUCTION

The year in review is the second of the Breast Cancer Task Force Program and its support through management and contracts by the Endocrine Evaluation Branch. During this year some progress has been made in carrying out objectives set up the previous year, and in clarifying and deepening plans for the future. The Task Force has continued to emphasize the need for better means of detecting the high risk patient and for extending the study of host-tumor relationships in order to define and attack practical problems. It plans to hold a three-day meeting in July 1968 for a critical review of its philosophy and program.

ORGANIZATION TO CARRY OUT BREAST CANCER TASK FORCE AIMS

The Task Force has recommended a program of identifying and screening high risk groups, of defining local or potentially curable disease, and of developing techniques for predicting the probability of recurrence of disease in human patients. It has also recommended studies on the biology of tumors to improve understanding of the factors that favor dissemination, and the development of animal model systems for studies on the effects of hormonal environment and chemotherapy. For implementation of these goals the Task Force looks to the cooperative clinical groups, two of which are represented on the panel, and to the General Laboratories and Clinics (NCI), the Chief of whose Endocrinology Branch is panel Chairman and the Chief of whose Endocrine Evaluation Branch is Executive Secretary of the panel. The Endocrine Evaluation Branch carries out the principal functions of management and program integration, through its contract program and liaisons with cooperative groups and NCI programs such as the Chemotherapy Program.

Collaboration with clinical groups is not limited to the two represented on the Task Force, but the latter have explicitly committed themselves to such collaboration. Both groups have advanced programs of their own to which compatible elements of the Task Force program are being added. The Cooperative Breast Cancer Group consists of members from 19 institutions, who have been carrying out studies on hormonal and nonhormonal agents in the treatment of advanced disease. Three of these institutions have now begun studies on primary breast cancer with emphasis on the prognostic and diagnostic aims of the Task Force, studies that require contract support as described later. The Surgical Adjuvants Breast Study Group, with members in 35 institutions, has been attempting to evaluate adjuvants—radiotherapy and oncolytic agents—in use with mastectomy, oophorectomy or other surgery. This group is now

planning studies of the Task Force-reiterated problem of evaluating simple and radical mastectomy, and some of its members will probably participate in the primary breast cancer protocol of the Cooperative Breast Cancer Group.

Central management of the Task Force Program is provided by the Endocrine Evaluation Branch, whose own contract program in chemotherapy and endocrine tumor biology has been supplemented for emphasis on projects involving breast cancer. The Branch consists of an Office of the Chief and two Sections (Table 1). Due to the small size of the Branch and the multifaceted nature of the program, two or more of these divisions usually share responsibility for a contract or project. Thus, the preclinical workup of agents for chemotherapy involves members and contracts in both sections and in the Office of the Chief, and contracts in either section dealing with clinical situations are managed under joint responsibility with the Coordinator of Clinical Activities in the Office of the Chief.

New Cooperative Clinical Research Supported by Contracts

Several of the Task Force objectives—identification of high risk groups, study of the biology of the tumor for understanding factors favoring dissemination, and development of techniques for predicting recurrence of disease—essentially constitute a plea for improved diagnosis and prognosis. A Task Force Subcommittee on Biological Determinants considered a variety of suggestions on endocrine and nonendocrine factors possibly affecting the development of breast cancer, and gave highest priority to studies on pathology and endocrinology in early breast cancer. A protocol for gathering data on clinical factors, including 13 urinary steroidal metabolites and several pathological features, was drawn up by a committee of the Cooperative Breast Cancer Group, and approved. It called for the study of approximately 300 patients per year, tissue and urine samples being taken before and at intervals after mastectomy for primary breast cancer. One objective of the study is to confirm or refute the prognostic value of the Bulbrook discriminant (an empirical index based on the level of two urinary steroids) in this trial situation.

Cooperating clinicians in three institutions with appropriate surgical facilities were chosen to furnish patient material according to the protocol. Contract support handled by the Endocrine Evaluation Branch consists of the following: two laboratories for doing steroid determinations on urine specimens (Worcester Foundation for Experimental Biology, Research Foundation for Mental Hygiene) and a statistical center (at Louisiana State University, already engaged in keeping medical records for the Breast Group). Arrangements for the performance of the functions of a central pathology laboratory were completed under a contract with the Boston University Medical School early in 1968. These contracts are handled by the Section on Biological Activities of Hormones. Day to day coordination of the studies resides also in this Section. During the past year extensive "blind" experiments have been carried out to determine the precision of the steroid determinations as effected through automated procedures. The protocol studies on primary breast cancer got under way in February, 1968.

As the facilities for these types of studies are expanded it is expected that other cooperative institutions will join in the primary breast cancer studies, and protocols will be drawn up for the inclusion of patients at the stage of disease where oophorectomy, adrenalectomy or hypophysectomy is required. The prospects for such expansion are excellent, since both contractors carrying out steroid determinations are progressing rapidly towards total automation.

Task Force support for improved diagnosis and prognosis is evidenced also in other contracts. Investigators at the University of Chicago Medical School are studying the "estrophiles" from cancer biopsy material to see whether estradiol uptake as a presumptive indicator of hormonal dependency will predict the patient's response to adrenalectomy. This project has been slowed by the inadequate case load, but recent improvement of technique has made it possible to utilize frozen material, and arrangements are being made for increased cooperation with surgeons outside the local area. Other prognosis-oriented contracts now being evaluated include two proposals for the study of preneoplastic lesions (as seen in the DeOme mouse studies) in rats, dogs and human beings, and proposals for the study of patterns of heat emission from human breast tumors. Finally in this connection it is appropriate to mention the prospective mammography studies carried out by the Hospital Insurance Plan under a contract managed by the Biometry Branch (Etiology) and approved for renewal this year by the Breast Cancer Task Force Contract Review Committee: arrangements have been made with the Endocrinology Branch to carry out assays for pituitary gonadotropins on the sera of key series of patients, including patients who develop breast cancer and controls.

Hormonal Therapy and Chemotherapy

In addition to the use of surgery—mastectomy, oophorectomy, adrenalectomy—in the attempt to cure or palliate breast cancer at various stages, the Task Force also recognizes the need for improved use of drugs and hormones. Thus, it set up a special committee to advise the Surgical Adjuvants Breast Cancer Study Group on the use of a drug for prophylactic purposes after mastectomy. (The committee recommended a low dosage of methotrexate.) The Task Force also supports the efforts of the Cooperative Breast Cancer Group in their trials with novel steroids, dosage response studies of drugs of known effectiveness, and combinations of hormones and cytotoxic agents. The Endocrine Evaluation Branch backs up this support with a program of preclinical chemotherapy that emphasizes hormonal drugs (since CCNSC handles the cytotoxic agents) and endocrine-related tumor systems for testing purposes.

The chemotherapy program involves all parts of the Branch. The Compound Supply Unit largely through a contract with the Upjohn Company provides (mostly endocrine) compounds for animal tests as well as for clinical trials. It also provides compounds of novel structure made by Research Triangle Institute, or in some cases by academic donors. In 1967 this Unit received 172 compounds, of which 147 were steroids and 25 nonsteroids; about one-fifth of these (35, 30 and 5 respectively) were "repeats" ordered for further testing. Nine repeat orders for clinical steroids were filled.

All new compounds are entered into the CCNSC accessions and distributed for study by Branch contractors, CCNSC or other interested parties. Bioassays are carried out by the Section on Biological Activities of Hormones: these include conventional steroid bioassays for initial screening and special tests, such as one for erythropoietic effects, for compounds of clinical interest. The bioassays are done at the Mason Research Institute, in Worcester. Tumor tests are carried out by the Endocrine-Related Tumor Section, primarily with rat mammary tumor systems but also with tumors originating in other tissues. Further details on these tests are given below.

For the past several years the Research Triangle contract has been making compounds in which cholesterol and other steroids were united with cytotoxic agents. Within the last year one of these, phenestrin, has shown unusually good effects in several of the endocrine-related tumor systems. This compound is a cholesterol ester of p-di-(2-chloroethyl)-aminophenylacetic acid; it was first synthesized several years ago by Russian investigators and may have received some clinical trial, but the literature on them is scant. During the next year these new compounds will be studied in all the biologic systems at the disposal of the Branch, in the hope that it may become possible to offer the clinicians some new drugs better than the hormones or cytotoxic agents now used in breast cancer.

Animal Tumor Models, Test Systems and Biological Studies

The original Study Group on Breast Cancer recommended, among other things, "the development of an optimal animal model that would facilitate the study of the effects on the tumor of manipulations of the hormonal environment." This in fact has been the main purpose of the Section on Endocrine Related Tumors. Through its contracts this Section has studied a large number of tumor systems originating in endocrine tissues or hormone-target tissues, or showing some endocrine dependency. These have been used, according to their peculiarities, for testing compounds or for a variety of biological studies. The Staff has never considered that a single tumor system could model for a multifaceted disease like breast cancer, and the Subcommittee on Animal Tumors of the Task Force has encouraged continuation of work with various types of mammary tumor as well as tumors of other origins. Some examples are as follows:

At the Southern Research Institute studies have been initiated to determine doubling time and cell generation time in the hydrocarbon-induced mammary rat tumors, and to determine the feasibility of using the Brennan technique for obtaining single (per host) carcinogen-induced tumors.

At the University of Miami, eight different lines of DMBA-induced transplantable mammary systems in Fischer 344 inbred rats have been tested for response to ovariectomy before or after tumor implantation. In two of the systems estrogen dependency was apparent: the tumors regressed after ovariectomy and the animals survived longer than untreated controls.

At the Mason Research Institute, where the bulk of the antitumor tests are carried out, a study is under way to determine how the age of the host (Fischer 344 rat) affects tumor growth and response of the #13762 mammary tumor to therapy.

At Squibb Research Institute a comparative study of enzymes and nucleotides has been made in a spontaneous and transplantable endocrine-responsive mammary adenocarcinoma and in a methylcholanthrene-induced and transplantable, endocrine-unresponsive mammary adenocarcinoma, both in Wistar rats.

At the Hospital for Joint Diseases in vivo and in vitro (organ cultures) studies of a metastasizing mammotrope tumor are being carried out, in Wistar-Furth inbred rats. Organ culture studies of a mammary tumor in the same host showed that the tumor grows readily without insulin or cortisol, but control mammary tissue does not.

Finally, in an effort to clear the way for new contract approaches, the Breast Cancer Task Force sponsored a two-day working conference on the micro-anatomy and preneoplastic structures of mammary tissues. The example of the DeOme "hyperplastic alveolar nodule" in the mouse and the possible usefulness of whole mounts of dog and human mammary glands were key subjects in the discussions. Later, two contracts were approved for studies directed at the finding of preneoplastic structures, in beagle dogs at the Davis campus of the University of California, and in human breast tissues at the University of Oregon Medical School.

The Viral Etiology in Breast Cancer

The interest of the Task Force in the possible viral etiology in breast cancer is served by four contracts of the Viral Oncology Branch (Etiology). These cover studies on animal and human breast tissues through the use of electron microscopy at Charles Pfizer and Company, through the use of mammary tumor virus assays at Georgetown University and Melpar, Inc., and through intensive virological study of human breast tissue and milk, at the Institute for Medical Research in New Jersey. Dr. W. Ray Bryan, the Project Officer, and Dr. D. Jane Taylor of the Endocrine-Related Tumor Section, are cooperating in a systematic search for viruses in the mammary tumors used by EEB contractors and colleagues. The tissues are sent to the Pfizer installation for study.

Fields Covered by Contracts of the Branch

A rough breakdown to show fields and funding (in round figures) of contracts entering the new fiscal year would be as follows: 2 contracts for compound preparation and synthesis ($200,000), 6 contracts for endocrine-related tumor screening and research ($775,000), 1 contract for endocrine bioassay and physiological research ($175,000), 3 contracts for exploratory prognostic studies involving steroids ($300,000), 1 contract for medical records and statistics ($110,000); and the following new contracts: 1 for a central pathology laboratory for the cooperative clinical studies ($45,000), 1 for study of preneoplastic lesions in dogs ($80,000) and in human beings ($80,000),

and 1 for a study of heat emission patterns in breast tumors (amount to be determined). Thus there will be 16 contracts costing approximately 2.0 million dollars handled by this Branch. Supplementing this from the standpoint of Task Force interest but only partly supported by Task Force funds are 5 contracts handled by Etiology—4 in the solid tumor-virus category (totalling $300,000) and the prospective mammography study ($500,000).

SUMMARY OF PROGRESS AND FORECAST OF DIRECTION

In the view of the Staff the following represent important advances during the last year:

1. For the first time, patients are being entered in the Primary Breast Cancer Treatment study. This culminates years of planning by the Cooperative Breast Cancer Group and the Endocrine Evaluation Branch for a workable protocol and adequate contract support.

2. There has been a revival of interest in the chemotherapy program, leading to the hope of finding agents for breast cancer that differ from the traditional types of hormone and cytotoxic agent.

3. The contract profile of the program has been broadened, largely in ways that intensify biological probing into clinical fields. Thus the pathology lab set up in connection with the Primary Breast Cancer Treatment study will make it possible to look prospectively at the histological character of tumors, lymph nodes, and blood vessel invasion, in relation to endocrine parameters, clinical state and survival of the patients.

4. Two new contracts will push a search for preneoplastic lesions in dog and human breast material, and others will attempt to relate the heat emission pattern of breast tumors to response to therapy.

5. The search for viruses in breast tumor material was extended through a contract for the examination of human tissues and milk from a population with high breast cancer incidence, and, through cooperation of contractors and colleagues in and out of NCI, to screen a large number of animal tumor systems for virus.

Forecast of future changes of direction is hazardous, especially in view of the scheduled review of the Breast Cancer Task Force this summer. On the basis of the established program there is reasonable hope for continuation and improvement of the long term clinical studies. One may expect to see entry of 300 patients in the study on Primary Breast Cancer Treatment, aimed at relating pathology and urinary steroids to morbidity and survival; introduction of some completely new types of drug for the treatment of advanced breast cancer, and completion of dosage response studies with standard compounds; a new study on prophylactic use of methotrexate after

mastectomy; and further prospective data on mammography in relation to the timely diagnosis of breast cancer. The search for preneoplastic lesions in dog and man will be started but must also be regarded as long-term.

The search for viruses in experimental animal mammary tumors and human cancer and milk are under way, and are expected to yield interesting results this year. Within a year also one may expect that the tumor heat emission study will demonstrate the feasibility (or lack of it), of a method for rapid detection of a tumor's response to treatment. Finally, the Staff is especially optimistic about the possibility that a method will be perfected to determine the estrogen dependency of a tumor through the use of biopsied material, as an indicator of probable response of the patient to ablative therapy.

TABLE 1

ORGANIZATION OF THE ENDOCRINE EVALUATION BRANCH

Office of the Chief. Function: General direction, coordination of program through Task Force, Review Committee and clinical groups. Dr. E. P. Vollmer, Chief; Dr. Stella Booth, Coordinator for Clinical Activities; Miss Barbara Stevenson, Program Analyst in charge of drug supply and distribution; Mrs. E. Weinel and Mrs. H. Donovan, Secretaries.

Section on Endocrine-Related Tumors. Function: Research on the biology of endocrine-related tumors and development of model tumor systems; experimental chemotherapy for preclinical information on potential antitumor agents. Dr. D. Jane Taylor, Head; Mr. C. Piczak, Biologist; Mrs. S. Bokar, Secretary; Mrs. M. Thomas, Clerk-typist.

Section on Biological Activities of Hormones. Function: Bioassay and research in endocrine physiology for use in chemotherapy and structure-function studies; exploratory diagnostic and prognostic research and the related medical records and statistical control. Dr. Ihor J. Masnyk, Head; Miss L. Trench, Chemist; Mrs. A. Moss, Secretary.

Table 2: BIBLIOGRAPHY

from

Contracts of the Endocrine Evaluation Branch

Contract #62-171 - American Medical Center at Denver

1. Jacobs, B.B. and Huseby, R.A.: Growth of tumors in allogenic hosts following organ culture explanation. Transplantation 5: 410-419, May 1967.

2. Jacobs, B.B. and Huseby, R.A.: Neoplasms occurring in aged Fischer rats, with special reference to testicular, uterine and thyroid tumors. Journal of the National Cancer Institute 39: 303-309, Feb. 1967.

Contract #62-106 - Columbia University

1. Kim, U.: Factors controlling metastasis of experimental breast cancer. Cancer Research 26: 461-463, 1966.

Contract #64-46 - Hospital for Joint Diseases

1. Ambellan, E. and Hollander, V.P.: A simplified assay for RNase activity in crude tissue extracts. Analytical Biochemistry 17: 474-484, 1966.

Contract #64-495 - Jefferson Medical College

1. Curti, J.T., Rupp, J.J., and Cantarow, A.: Influence of 5-Fluorouracil on thyroid function. Proceedings of the Society for Experimental Biology and Medicine 125: 1125-1126, 1967.

Contract #65-6 - Mason Research Institute

1. Smith, E.R.: The stimulation of canine prostatic secretion by sympathomimetic amines. Journal of Pharmacology and Experimental Therapeutics 156: #2, 1967.

Contract #64-80 - University of Miami

1. Dunning, W.F., Curtis, M.R., Stevens, M.L., and Dumenigo, F.: Five transplantable leukemias in the Fischer rat, and their responsiveness to steroids. Cancer Research 27: Part 2, 696-727, June 1967.

Contract #4351 - Research Triangle Institute

1. Dudley, K.H., Corley, R.C., Miller, H.W., and Wall, M.E.: Flavonoids. III. Studies on the synthesis of 2,4-Dialkyl-7-acetoxy-4'-methoxy-Δ^3-isoflavenes. Journal of Organic Chemistry 32: 2312, 1967.

2. Dudley, K.H., Miller, H.W., Corley, R.C., and Wall, M.E.: Flavonoids. IV. A novel clemmensen reduction. The direct conversion of 2-Alkyl-isoflavones to 2-Alkyl-Δ^3-isoflavones. Journal of Organic Chemistry 32: 2317, 1967.

3. Dudley, K.H., Miller, H.W., Corley, R.C., and Wall, M.E. Flavonoids. V. Thiation of isoflavones. Journal of Medicinal Chemistry 10: 985, 1967.

4. Rosenthal, D., Lefler, C.F., and Wall, M.E.: Steroids - LXXVIII. The lead tetraacetate oxidation of a 5-hydroxy-B-norsteroid. Tetrahedron 23: 3583-3600, 1967.

5. Rosenthal, D.: The mechanism of the lead tetraacetate oxidation of a B-norsteroid. Experientia 23: 686, 1967.

Contract #2395 and 65-1050 - E. R. Squibb and Sons

1. Hilf, R., Bell, C. and Michele, I.: Influence of the mammotropic tumor MtTF4 on the growth and biochemistry of the R3230AC mammary carcinoma and mammary glands. Cancer Research 27: 482-489, 1967.

2. Hilf, R., Michele, I., and Bell, C.: Biochemical and morphological responses of normal and neoplastic mammary tissue to hormonal treatment. Recent Progress in Hormone Research 23: 229-295, 1967.

3. Hilf, R., Goldenberg, H., and Bell, C.: Effect of Actidione (Cycloheximide) on estrogen-induced biochemical changes in R3230AC mammary tumors, uteri and mammary glands. Cancer Research 27: 1485-1493, August 1967.

4. Hilf, R., Michele, I., and Bell, C.: Biochemical and morphological responses of normal and neoplastic mammary tissue to hormonal treatment. Recent Progress in Hormone Research Vol. XXIII, 229-295. Academic Press, Inc., New York, N.Y. (1967).

5. Lerner, L.J. and Hilf, R.: Biological activities of steroids and their relationship to breast cancer therapy. In: Current Concepts in Breast Cancer (A. Segaloff, K. K. Meyer and S. DeBakey, Eds.), 80-93. The Williams and Wilkins Co., Baltimore, Md. (1967).

6. Hilf, R. and Lerner, L.J.: Experimental Mammary Tumors: Biochemical responses to hormonal treatments. In: Current Concepts in Breast Cancer (A. Segaloff, K. K. Meyer and S. DeBakey, Eds.), 247-259. The Williams and Wilkins Co., Baltimore, Md. (1967).

ANNUAL REPORT OF THE ASSOCIATE SCIENTIFIC
DIRECTOR FOR CLINICAL RESEARCH
NATIONAL CANCER INSTITUTE

July 1, 1967 through June 30, 1968

For this annual report, I would like to be brief and to mention a few administrative changes and to record a few observations about the changing pattern of research.

The administrative changes concerned are the appointment of Dr. Marvin A. Lutzner as Chief of the Dermatology Branch and Dr. Thomas A. Waldmann as Head of the Immunophysiology Section, a new section in the Metabolism Branch.

In the last year, the experimental surgical facility in Building 14D was completed and put into operation. The principal research observation is the continuing development of a competency in the basic sciences by clinical investigators. This change has been most dramatic in the last two years in the Surgery Branch. There are now a number of investigations in the Surgery Branch that are concerned with applying the tools and techniques of the basic sciences to the study of clinical problems. There has been a growing interest in studies that are of immunological or virologically oriented or that deal with the changing physiology of the surgical patient.

Within the other clinical branches, the same pattern is emerging of clinical research that is both patient oriented and a complementary research in the laboratory and the experimental animal. Generally, the laboratory studies are designed to develop new ideas before their application in man.

While there is not a need for a large amount of additional space in the Clinical Center, the Dermatology and the Surgery Branch have a requirement for additional animal rooms. The Immunology Branch will have made available to it additional space on the fourth floor. In general, the numbers of persons now available are sufficient, but there are scattered areas where additional personnel would be highly desirable. This is particularly true in the experimental surgical laboratory and in the Immunology Branch.

Serial No. NCI-15-000
1. NCI
2. GL&C
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Screening

Principal Investigator: Dr. Nathaniel I. Berlin

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Endocrinology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes__ No X

Serial No. NCI-15-001
1. NCI
2. GL&C
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Screening

Principal Investigator: Dr. Nathaniel I. Berlin

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Endocrinology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

Serial No. NCI-15-002
1. NCI
2. GL&C
3. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Part A

Project Title: Emergency Admissions

Principal Investigator: Dr. Nathaniel I. Berlin

Other Investigators: Physicians participating in patient care

Cooperating Units: Dermatology Branch, Endocrinology Branch, Immunology Branch, Medicine Branch, Metabolism Branch, Radiation Branch, and Surgery Branch

Man Years
Total:
Professional:
Other:

Project Description: This is a mechanism by which this necessary hospital function can be classified.

Part B included Yes___ No X

Part B Journal Vol. No.

Part A

SUMMARY STATEMENT
ANNUAL REPORT
Dermatology Branch - NCI
July 1, 1967 - June 30, 1968

The clinical and laboratory research program of the Dermatology Branch has continued along lines developed in recent years. The major research interests are: (1) clinical investigations of mycosis fungoides, other malignant skin diseases, related benign skin diseases and genodermatoses which predispose to malignancies, (2) electron microscopic studies of mycosis fungoides, other skin neoplasms, virus-induced skin diseases, genodermatoses, effect of poke weed mitogen in the production of plasmoblasts, (3) identification and study of a murine model for the Chediak-Higashi syndrome and Aleutian mink. Study of the polygenic effects on a single cytoplasmic organelle - the melanin granule - in coat color mutants of the mouse, (4) the behavior of normal and diseased human epidermis in tissue culture, (5) utilization of microbes in the development of assays for drugs of pharmacological interest and in the development of specific enzymes, (6) tissue culture studies of human lymphocytes, (7) synthesis of alkaloids, (8) the biochemistry of normal and pathological epidermis.

1. Clinical Investigations. Experience with intermittent Methotrexate therapy in patients with mycosis fungoides suggests that when weekly intramuscular injections of Methotrexate fail to produce remission, significant improvement can sometimes be achieved by manipulating the dosage-time schedule; that is, treating every 6, 5, 4 days or semi-weekly. Treating more frequently results in bone marrow toxicity. With this manipulation of time schedule, it has been possible in a series of thirty patients with mycosis fungoides to achieve complete remissions in 20% and regression of lesions in 60-75% of patients. Further, it has been found that the number of cells in mitosis (studied in biopsy material from a lesion), is proportional to the resistance of that lesion to therapy.

Plasma fractions directed against mycosis fungoides tumors are being bioassayed in cooperation with the Immunology Branch, NCI. Delayed hypersensitivity reactivity of these patients will be incorporated in the study.

The natural history, pathological features and current therapeutic approaches in patients with mycosis fungoides will be computerized and data processing techniques will be used in an attempt to analyze mechanisms operating in these patients.

2. Electron Microscopic Studies of Mycosis Fungoides, Skin Neoplasms, Genodermatoses, Transformation of Rabbit Epidermal Cells by Shope Papilloma Virus. Study of a Murine Model for Chediak-Higashi Disease. Cells with bizarre nuclei have been found in skin lymph nodes and/or blood from patients with mycosis fungoides and Sezary's Syndrome, using the electron microscope. To date these characteristic cells seem specific for these two closely related diseases and may prove

to be useful for diagnosis. An attempt is being made to determine whether the characteristic cell can be found in tissues from patients with parapsoriasis en plaque and parapsoriasis variegta (diseases which are considered to be prodromal to mycosis fungoides). Transformation of rabbit epidermal cells by Shope papilloma virus are being studied.

It has been found that the beige mouse, genotype bg bg, is the probable counterpart for the Chediak-Higashi and Aleutian mink syndrome. An electron microscopic study of embryonic melanin granules from these animals may shed light on what seems to be a genetically-induced granule defect.

3. Utilization of Microbes in the Development of Assays for Drugs of Pharmacological Interest. An organism has been isolated from soil which can utilize the drug Methotrexate as the sole carbon and nitrogen source. An enzyme which degrades Methotrexate has been isolated and is the basis for a new spectrophotometric assay for Methotrexate in the urine. Furthermore, the chemical properties of this enzyme have been determined. In addition, several specific enzymes - peptidases - have been isolated by this soil enrichment technique. These peptidases are being further characterized.

4. Tissue Culture Studies of Human Lymphocytes. Mechanisms concerned with control of mitosis, differentiation, function and potentialities of normal and pathologic cells with particular reference to lymphocytes and cells of the lympho-reticular system are being studied. A rapid centrifugation technique has been developed for the separation of neutrophils from lymphocytes and monocytes. These derived lymphocytes are still PHA transformable and the monocytes have retained their ability to phagocytize.

Lymphocytes in patients with chronic lymphocytic leukemia are known to be unable to respond to the blastogenetic activity of PHA. This impairment has been demonstrated to persist even when the culture period is extended to ten days, even in the presence of homologous plasma or mononuclear cells, and even in the presence of added L-asparagine to the culture. This supports the possibility that the cells themselves are inherently defective.

It has been found that cells with phagocytic function are not transformable by PHA and further it has been found that the presence of PHA does not influence the synthesis of RNA in phagocytic cells. Nor, was there any evidence for transfer of labelled RNA from these phagocytic cells to lymphocytes undergoing blastogenesis.

5. Synthesis of Alkaloids. Several new alkaloids have been synthesized and the conditions for, and rate of, their synthesis have been determined. Alkaloid biogenesis is being sought in mammals. Such biogenesis might be expected to involve naturally occurring compounds and known biochemical reactions. This project is being terminated.

6. Biochemistry of Normal and Pathological Epidermis. Human epidermal keratin is solubilized by trypsin. Further non-enzymatic solubilization takes place, possibly by "unwinding" of helical portions. Purification and characterization of the helical structure of "keratin" is being defined as to composition, molecular weight and relationship to the fibrous aggregate known to occur in

epidermal cells. The rate of DNA synthesis in chick embryo skin in chemically defined medium has been found to remain unchanged when the serum of patients with psoriasis is added to the medium.

7. Direct Measurements of Acetylsalicylic Acid (ASA) in Human Blood. Attempt is being made to develop a method for the direct measurement of ASA in body fluids. When this technique is achieved it will be used to correlate the levels of ASA in plasma with clinical responses (antirheumatic and anti-inflammatory effects).

Serial No. NCI-3600
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Clinical Investigations of Malignant Skin Diseases and of Benign Conditions Related Thereto

Previous Serial Number: Same

Principal Investigator: Harley A. Haynes, M.D.

Other Investigators: Marvin A. Lutzner, M.D., Joseph D. Croft, Jr., M.D. and Arthur R. Ugel, M.D.

Cooperating Units: Medicine Branch, NCI
Radiation Branch, NCI
Pathologic Anatomy Branch, NCI
Laboratory of Clinical Investigations, NIAID
Arthritis and Rheumatism Branch, NIAMD

Man Years (July 1, 1967 through June 30, 1968)

Total:	1.9
Professional:	1.9
Other:	0

Project Description:

Objectives:

To study malignant processes in the skin and to carry out investigative therapy of cutaneous malignant conditions; to study benign hyperplasia of the epidermis in relation to malignant hyperplasia of the skin and other organs.

Methods Employed:

Patients with cutaneous lymphoma, primarily mycosis fungoides, are studied. The mitotic activity of their lesions is estimated and the patients are treated with intermittent methotrexate. Those not responding to weekly doses are given the drug more frequently - either semi-weekly or every 5 days.

Major Findings:

In general, the degree of response to weekly methotrexate has varied

inversely with the mitotic count in the lesions. Some patients with high mitotic counts and poor responses to weekly methotrexate have had significantly improved responses on a semi-weekly schedule. Of 30 patients, the overall response has been 20% complete remission and 60% with 75% or better regression of lesions.

Patients not requiring systemic chemotherapy are treated with topical nitrogen mustard and/or topical corticosteroids.

In cooperation with the Medicine and Radiotherapy branches, a study of the immunologic reactivity of 100 untreated patients with Hodgkin's disease has shown that anergy is much less common than previously believed - 75% are able to develop delayed hypersensitivity to DNCB. Reactivity in stage I disease appears normal, while impairment is most common in stages III and IV. The relationship of immunologic reactivity to prognosis is currently being investigated.

Significance to Cancer Research:

Project directly concerned with study of, and therapeutic influences on, cancer and related conditions.

Proposed Course of Project:

Patients previously studied will be followed over the next several years.

Honors and Awards: None

Publications:

1. Waldorf, D. S., Haynes, H. A., and Van Scott, E. J.: Cutaneous hypersensitivity and desensitization to mechlorethamine in patients with mycosis fungoides lymphoma. Ann. Intern. Med. 67: 282-290, Aug. 1967.

2. Waldorf, D. S., Willkens, R. F., and Decker, J. L.: Impaired delayed hypersensitivity in an aging population. J.A.M.A. 203: 831, Mar., 1968.

3. Haynes, H. A., and Van Scott, E. J.: Therapy of mycosis fungoides. In Blank, H. (Ed.): Progress in Dermatology. New York, N.Y., Dermatology Foundation. (in press).

Serial No. NCI-3606
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Electron Microscopic Studies of Mycosis Fungoides, Sézary's Syndrome, Parapsoriasis and their Interrelationship; Study of the Effect of Poke Weed Mitogen in the In Vivo and In Vitro Production of Plasmoblasts; Search for and Study of a Murine Model for the Chediak-Higashi Syndrome and the Aleutian Mink; In Vitro Synthesis of Epidermal Proteins.

Previous Serial Number: Same

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Arthur R. Ugel, M.D.

Cooperating Units: Dr. Barbara E. Barker and Dr. Patricia Farnes, Department of Pathology, Rhode Island Hospital, Providence, Rhode Island (Poke Weed Mitogen Studies).
Dr. Carl T. Hansen and Miss Phyllis Phillips, Rodent and Rabbit Production Section, LA (Mouse Coat Color Mutant Studies).

Man Years (July 1, 1967 through June 30, 1968)

Total:	5.0
Professional:	1.5
Other:	3.5

Project Description:

Objectives:

(1) Electron microscopic studies of mycosis fungoides, Sézary's Syndrome, parapsoriasis and skin cancers. (2) Electron microscopic studies of the Shope papilloma virus and conversion of "verrucal" epidermal cells to neoplastic cells. (3) To study plasmacytoid changes in lymphocytes cultured in vitro in the presence of poke weed mitogen. (4) Study of a murine model for the human genetic disease - Chediak-Higashi syndrome. (5) Isolation of polysomes from keratin-producing tissues, synthesis of proteins in cell-free systems.

Methods Employed:

Electron microscopy of human, rabbit and murine tissues. The breeding of coat color mutants of mice and the development of "double" and "triple"

coat color mutants. Homogenization of epidermis and density centrifugation techniques for isolation of polysomes. Radioisotopic techniques in study of the incorporation of amino acids into epidermal proteins.

Major Findings:

(1) The characteristic cell previously found in tissues of patients with mycosis fungoides and Sézary's Syndrome has been observed now in twenty-four of twenty-eight mycosis fungoides patients studied and in four of four Sézary patients studied. Further, three cases of parapsoriasis en plaque and one case with parapsoriasis variegta have been studied and the characteristic mycosis fungoides cell has been found in all four of these patients - indicating the diagnostic potential of this characteristic cell.

(2) Skin of domestic rabbits was examined sequentially following the application of Shope papilloma virus. Although visible lesions developed within one week, no virus could be found (electron microscopically) in epidermal cells. In addition, squamous cell carcinoma lesions which developed 7 to 12 months after the application of the Shope virus in domestic rabbits were studied with the electron microscope. No recognizable virus was found. Further, changes have been noted in the transformed epidermal cell and these are being studied.

(3) Cells resembling plasma cells were found to be abundant in peripheral blood sampled from a child who accidentally ingested poke weed. Electron microscopic studies confirmed the presence of large amounts of rough endoplasmic reticulum in these cells. Also, it was observed that lymphocytes, cultured in vitro for 10 days in the process of poke weed mitogen, developed large quantities of rough endoplasma reticulum and appeared to be modulating towards plasma cells.

(4) A coat color murine mutant called beige, genotype (bg/bg), had been known to have giant melanin granules in hair, retina and choroid. We have found that these beige mice also have giant granules in their peripheral blood leukocytes. These findings of giant melanin granules and giant leukocyte granules in the beige mouse, homogenous for the autosomal recessive mutant gene (bg), suggests that it may be the murine counterpart for the Chediak-Higashi human and the Aleutian mink. Hence, may serve as a convenient murine model for these genetic syndromes.

(5) Using the methods of differential centrifugation, single ribosomes and small polysome units have been isolated from epidermal cells and identified in the electron microscope.

Significance to Cancer Research:

Search for virus in human tumors seems warranted in view of the evidence for virus etiology in a number of animal tumors; skin cancers are most available for studying because of ease in obtaining biopsies. The characterization of an abnormal cell in mycosis fungoides should aid in diagnosing the disease

in its earliest stages. Study of genetic human disorders which predispose to cutaneous malignancies might reveal clues as to the pathogenesis of cancer. The understanding of the role of the papilloma virus in producing skin cancers in rabbits might be enhanced by studying the ultrastructural changes produced in the epidermal cell. The study of the transformation of lymphocytes from cells to the plasma cell served by poke weed mitogen might shed light on the role of the lymphocyte and immunologic mechanisms. Study of the gene influence on melanin granule formation might provide insight as to polygene influences on the formation of a specific cytoplasmic orgenelle the melanin granule.

Proposed Course of Project:

It is proposed that the above stated objectives be pursued. Patients with rare genodermatoses will be sought and studied. Rabbits will be inoculated with papilloma virus and the resulting warts and tumors will be studied. Melanin granules from various coat color mutants of mice will be examined electron microscopically. Melanin granules from "double" and "triple" mutants will also be examined in an attempt to determine polygenic effects on a cytoplasmic orgenelle - the melanin granule.

Honors and Awards: None

Publications:

1. Aaronson, C. M., and Lutzner, M. A.: Epidermodysplasia verruciformis and epidermoid carcinoma. J.A.M.A. 201: 775-777, Sept. 1967.

2. Flaxman, B. A., Lutzner, M. A., and Van Scott, E. J.: Cell maturation and tissue organization in epithelial outgrowths from skin and buccal mucosa in vitro. J. Invest. Derm. 49: 322-332, 1967.

3. Lutzner, M. A., Lowrie, C. T., and Jordan, H. W.: Giant granules in leukocytes of the beige mouse. J. Heredity. 58: 299-300, Nov.-Dec. 1967.

4. Flaxman, B. A., Lutzner, M. A., and Van Scott, E. J.: Ultrastructure of cell attachment to substratum in vitro. J. Cell Biol. 36: 406-410, 1968.

5. Flaxman, B. A., and Van Scott, E. J.: Keratinization in vitro of cells from a basal cell carcinoma. J. Nat. Cancer Inst. 40: 411-422, 1968.

6. Lutzner, M. A., and Jordan, H. W.: Ultrastructure of an abnormal cell in Sézary's Syndrome. Blood (in press).

7. Barker, B. E., Lutzner, M. A., and Farnes, P.: Ultrastructural properties of pokeweed stimulated leukocytes in vivo and in vitro. Monograph, Univ. of Iowa, Third Leukocyte Culture Conference (in press).

Serial No. NCI-3609
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Utilization of Microbes in the Development of Assays for Drugs of Pharmacological Interest.

Previous Serial Number: Same

Principal Investigator: Carl C. Levy, Ph.D.

Other Investigators: Peter Goldman, M.D.

Cooperating Units: NIAMD

Man Years (July 1, 1967 through June 30, 1968)

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives:

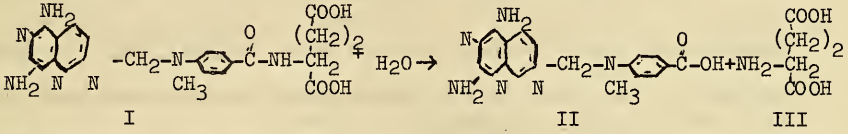
To isolate microorganisms from soil which can utilize various drugs as the sole source of carbon and/or nitrogen and to extract from these organisms enzymes which may be used to assay for the compounds.

Methods Employed:

In collaboration with Dr. Peter Goldman of NIAMD, the utilization of microbial agents as a means of developing specific assays for specific compounds of pharmacological interest is being studied. The drug of interest together with about a gram of garden soil is placed in a flask containing a simple salt solution, and the flask shaken for 2 to 3 days at 30°. The drug serves as the source of carbon and/or nitrogen and any organism growing in response to it is isolated in pure culture by standard microbial techniques. The microbe is then grown in larger quantities and the resulting cell mass disrupted by sonic osculation. If the enzyme isolated after cellular disruption attacks the particular drug of interest, then the nature of this attack can be used both in a qualitative and quantitative manner. Qualitative, in the sense that enzymatic activity indicates that the compound or one similar to it is present and quantitative in that, the rate in which the new product is formed can serve as a measure of the amount of compound present originally.

Major Findings:

Thus far an organism has been isolated from soil which can utilize the drug methotrexate as the sole carbon and nitrogen source. An enzyme obtained from the organism has been shown to catalyze the degradation of methotrexate in accord with the following equation:



I II III

For every mole of methotrexate (I) hydrolyzed; one mole of 2,4 diamino, N^{10}Methyl pteroic acid (II) and a mole of glutamic acid (III)is formed. This attack can be followed spectrophotometrically by recording the decrease in absorbance of methotrexate at 320 mμ. An assay based on this procedure has been used to measure the amount of methotrexate in urine. The hydrolytic attack on the N-Benzoyl-L-glutamic acid moiety of methotrexate suggested that the enzyme was a peptidase. This has been confirmed, and the properties of the enzyme more completely elucidated. Three additional enzymes have been isolated employing these techniques. These enzymes were hydrolyzed folic acid analogues containing aspartic acid, alanine and phenylalanine in the position normally occupied by glutamic acid.

Significance to Cancer Research:

The value of developing other means of assaying drugs used in cancer chemotherapy needs, I think, little elaboration. An ancillary benefit derived from this type of study is general knowledge of the potential metabolism of these drugs. In a complex animal system, the elaboration of metabolic relationships often proves difficult; whereas in a simpler biological system (e.g. bacterial) knowledge of the metabolic pathway can offer clues to the pathways in the animal. Thus the microorganism serves not only to measure the amount of drug present in biological fluids, but perhaps more importantly, serves as a model in understanding the metabolism of the compound.

Proposed Course of Project:

A. We shall continue the study of a number of drugs of interest in cancer chemotherapy and attempt to isolate organisms which will degrade them.

B. The metabolism of these drugs will be studied in the hope that some light will be shed on their metabolism in higher animals.

C. The peptidase nature of the enzyme hitherto described will be studied in more detail.

Honors and Awards: None

Publications:

1. Goldman, P., and Levy, C. C.: The microbiological preparation of pteroic acid. Biochem. Preparations (in press).

2. Goldman, P., and Levy, C. C.: Bacterial peptidases II. Enzymes specific for N-Acyl linkages to aspartate. J. Biol. Chem. (in press).

3. Goldman, P., and Levy, C. C.: Enzymes which hydrolyze analogues of folic acid. Biochem. Pharmacol. (in press).

Serial No. NCI-3607
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Tissue Culture Studies of Human Lymphocytes

Previous Serial Number: Same

Principal Investigator: Jay H. Robbins, M.D.

Other Investigators: William R. Levis, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	2.2
Professional:	1.2
Other:	1.0

Project Description:

Objectives:

To investigate the mechanisms concerned with the control of mitosis, differentiation, function, and potentialities of normal and pathologic cells, with particular reference to the lymphocyte and cells of the lymphoreticular system.

Methods Employed:

Human peripheral blood lymphocytes are cultured in vitro and stimulated to transform into large blastoid cells by factors such as phytohemagglutinin and antigens. This phenomenon of blastogenesis is evaluated by the morphologic and biochemical changes which the lymphocytes undergo. The mechanisms by which these blastogenic factors act and the culture conditions required for blastogenesis will be determined. The blastogenic factors will be purified, and their sites of action will then be determined by immunologic, microscopic and other techniques. Particular study will be made of cultures in which blastogenesis is impaired, for example, in cultures obtained from patients with certain diseases such as chronic lymphocytic leukemia and Hodgkin's disease, in an attempt to elucidate the nature of the impairment.

Patient Material:

Lymphocytes are obtained from hospitalized patients and out-patients

Major Findings:

1) A centrifugation technique for the removal of polymorphonuclear leukocytes from the mononuclear cells (lymphocytes and monocytes) of heparinized human peripheral blood has been studied and utilized. Within five minutes this technique yields plasma containing mononuclear cells contaminated less than 1 to 2% with polys. The lymphocytes so obtained are suitable for tissue culture studies, for they respond normally to blastogenic stimuli such as phytohemagglutinin (PHA), specific antigens, and homologous leukocytes. The monocytes so obtained develop into macrophages which exhibit phagocytic activity. The technique is the simplest available which permits the removal of polys from the mononuclear cells in so short a time and without the addition of foreign particles or chemicals.

2) Lymphocytes from the peripheral blood of patients with chronic lymphocytic leukemia (CLL) have been studied in tissue culture. The majority of these lymphocytes do not respond in a normal manner to the blastogenic activity of PHA. This impairment has now been demonstrated to persist even when the culture period is extended to 10 days and even in the presence of homologous plasma or mononuclear cells from healthy individuals. Addition to the cultures of L-asparagine also failed to improve the blastogenic response to PHA. These results indicate that it has not yet been possible to repair the deficit responsible for the impaired blastogenic response of these leukemia cells and support the possibility that these cells may have an inherent defect.

3) The only cell to be shown conclusively to undergo blastogenesis in the presence of PHA is the small lymphocyte. In an effort to determine if another cell can be similarly influenced, the response to PHA of the phagocytic cells in human peripheral blood has been studied. Our results indicated that none of these cells with phagocytic function had been stimulated by PHA to synthesize DNA. Other blastogenic factors, such as specific antigen and homologous leukocytes, also failed to stimulate DNA synthesis in cells with phagocytic function.

4) Autoradiographic studies have been undertaken to determine if blastogenic factors influence the synthesis of RNA in phagocytic cells of human peripheral blood in tissue culture. No effect was found. The cells synthesized RNA in the presence or absence of the blastogenic factors. The time course and locations of this synthesis were studied, and it was found that the H^3-label and uridine-5-H^3 appears first in the nuclear region and later in the cytoplasm. No evidence of transfer of label to lymphocytes undergoing blastogenesis was detected.

Significance to Cancer Research:

The studies are concerned with the control of cell division and differentiation in cells involved in normal and malignant processes.

Proposed Course of Project:

Continuation of research as indicated in the foregoing.

Honors and Awards: None

Publications: None

Serial No. NCI-3602
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Biochemistry of Normal and Pathological Epidermis

Previous Serial Number: Same

Principal Investigator: Simon Rothberg, Ph.D.

Other Investigators: Carl E. Kierney, M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total	3
Professional:	2
Other:	1

Project Description:

Objectives:

To study keratinization in epidermal tissue. To identify and characterize the protein or proteins synthesized by these epidermal tissues for the orderly formation of its specific "keratin". To study the cellular influences in epidermal tissue that control the synthesis of epidermal proteins which in different tissue results in the production of different "keratins". To identify and characterize the in vivo stimulants and inhibitors of mitotic activity and DNA synthesis in the epidermis.

Methods Employed:

Various techniques to enzymatically solubilize insoluble epidermal keratin have been extended. Soluble protein products (SPP) were obtained from keratin under conditions in which a minimal number of four proteins were obtained. By comparison of disc electrophoresis patterns (14% polyacrylamide in 8 M urea) of these proteins before and after additional trypsin action 3 of these 4 proteins were found to be derived from a single common protein.

Column Chromatography on D-32, Sephadex G-75, and Bio-gel-100 have verified the similarities between these proteins in size, shape, charge and their normal ability to aggregate under salt or buffer conditions. Some resolution has been obtained when column runs were made in 8 M urea buffer solutions.

Organ culture was carried out using 14 day chick embryo skin and culturing in BGJ-modified, a chemically defined medium. The C^{14}-Thymidine was added to follow both normal DNA synthesis and synthesis after the addition of various agents. DNA was isolated and quantitated by a modification of the method of Burton, and the radioactivity counted in a liquid scintillation counter.

Major Findings:

The soluble protein products (SPP) obtained after mild enzymatic treatment of insoluble epidermal keratin are qualitatively similar to the "unwind" SPP. These proteins have been shown to be related to each other and derived from a common protein. Because of the intrinsic aggregate nature of epidermal proteins, separation of these proteins by column chromatography or disc electrophoresis requires an 8 M urea buffer solution.

The rate of DNA synthesis in chick embryo skin grown in chemically defined medium is unchanged when serum of patients with psoriasis was added to the medium.

Significance to Cancer Research:

The evidence of enzymatic epidermal protein solubilization, the nature of these proteins and how they "unwind", and the relationship between the proteins solubilized and their helical structure permits a preliminary model for keratin to be made. From this model a better understanding of keratin and how it relates to epidermal carcinogenesis is possible.

Methodology for measuring DNA synthesis in chick embryo skin in a chemically defined medium has been established and can now be used as a model for testing the influences of various agents on DNA synthesis.

Proposed Course of Project:

Human epidermal proteins solubilized under a variety of enzymatic conditions will be purified and characterized as to helical structure, composition, molecular weight and relationship to the fibrous aggregate ("keratin"). Similar characterization of epidermal proteins from other sources will be made. With the present methodology DNA synthesis of embryonic skin in organ culture will be further investigated. In vivo substances which are inhibiting or stimulating DNA synthesis will be isolated, characterized, and tested in vitro.

Honors and Awards: Travel award to present paper at Fifth International College of Experimental Dermatology, Palermo, Italy, March 28-30, 1968.

Publications:

1. Rothberg, S., and Ekel, T.: Mitotic activity in the chick embryo epidermis. Nature, 216: 1352, Dec., 1967.

2. Rothberg, S., and Axilrod, G. D.: Proteolytic action on insoluble epidermal "keratin" and the nature of the epidermal proteins solubilized. Biochem. Pharmacol. Suppl., 269-270, Mar., 1968.

3. Rothberg, S. and Axilrod, G. D.: The action of trypsin on insoluble epidermal keratin and the nature of the epidermal protein solubilized. Biochemical Medicine, (in press).

4. Rothberg, S.: Epidermal keratin and keratinization. Proc. of the Fifth International Symposium of Experimental Dermatology, (in press).

Serial No. NCI-3608
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Synthesis of Alkaloids

Previous Serial Number: Same

Principal Investigator: Jay H. Robbins, M.D.

Other Investigators: None

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	.8
Professional:	.8
Other:	0

Project Description:

Objectives:

To investigate the conditions under which isoquinoline and β-carboline alkaloid synthesis occurs in vitro.

Methods Employed:

Postulated precursors of certain alkaloids are permitted to react in vitro under defined and controlled conditions of pH, temperature, etc., and the rate of alkaloid synthesis is determined by suitable chemical analyses.

Major Findings:

Several new alkaloids have been synthesized and the conditions for, and the rate of, their synthesis have been determined.

Significance to Cancer Research:

The discovery of alkaloid biogenesis in mammals would, in general, be a finding of fundamental significance. Such biogenesis can reasonably be expected to involve many natural compounds and known biochemical reactions which are now known to be essential for the metabolism of all cells, normal as well as malignant.

Proposed Course of Project:

Termination.

Honors and Awards: None

Publications: None

Serial No. NCI-3611
(Also NCI-5009)
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunologic Investigations in Mycosis Fungoides

Previous Serial Number: None

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Ronald B. Herberman, M.D., John L. Fahey, M.D., G. Nicholas Rogentine, Jr., M.D. and Joseph D. Croft, M.D.

Cooperating Units: Immunology Branch, NCI

Man Years (July 1, 1967 through June 30, 1968)

Total:	.2
Professional:	.2
Other:	0

Project Description:

Objectives:

Protccols for the study of delayed hypersensitivity reactions to human tumors and for bioassay of plasma fractions against malignant tumors have previously been submitted in detail. As part of these investigations, patients with mycosis fungoides will be studied to determine their ability to become sensitized to DNCB.

Methods Employed:

An attempt will be made to sensitize patients with mycosis fungoides to 2,4-DNCB. A sensitizing dose of 2,000 gamma of DNCB dissolved in 0.1 cc acetone will be placed on a 2 cm. diameter patch of normal skin and occluded with a band-aid for two to three weeks. Challenge doses of 100 gamma and 50 gamma of DNCB, each dissolved in 0.1 cc acetone and placed on a 2 cm. diameter patch of normal skin occluded with band-aids, will be inspected for evidence of positive delayed hypersensitivity reactions (erythema, erythema and edema or erythema, edema and vessiculation) after 48 hours and 72 hours. The challenge doses will be applied at the onset of testing (to determine whether patients are sensitive prior to the sensitizing dose) and two to three weeks after application of the sensitizing dose (to determine whether patients have become sensitive by the sensitizing dose).

Major Findings: None

Significance to Cancer Research:

The present study will be undertaken to investigate the possible role of delayed hypersensitivity in the response of patients to their own tumor. The utilization of DNCB is one of the parameters for the measurement of delayed hypersensitivity.

Proposed Course of Project:

Continuation along lines as outlined.

Honors and Awards: None

Publications: None

Serial No. NCI-3612
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Mycosis Fungoides: Natural History, Pathological Features, and Current Therapeutic Approaches. Data Processing and Computer

Previous Serial Number: None

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Joseph D. Croft, Jr., M.D.

Cooperating Units: None

Man Years (July 1, 1967 through June 30, 1968)

Total:	.2
Professional:	.2
Other:	0

Project Description:

Objectives:

To review our experience with patients who have mycosis fungoides.

Methods Employed:

Charts will be reviewed of all patients seen by the Dermatology Branch with the diagnosis of mycosis fungoides. Data process methodology will be employed and such information will be computerized.

Major Findings:

None

Significance to Cancer Research:

Purpose is to provide additional information about the natural history and therapeutic approaches in mycosis fungoides.

Proposed Course of Project:

Continuation along lines as outlined.

Honors and Awards: None

Publications: None

Serial No. NCI-3610
(Also NIAMD-ARB-23C)
1. Dermatology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Direct Measurements of Acetylsalicylic Acid in Human Blood

Previous Serial Number: None

Principal Investigator: Marvin A. Lutzner, M.D.

Other Investigators: Peter Goldman, M.D. and Joseph D. Croft, Jr., M.D.

Cooperating Units: NIAMD

Man Years (July 1, 1967 through June 30, 1968)

Total:	.2
Professional:	.2
Other:	0

Project Description:

Objectives:

Precise data identifying the specific antirheumatic and anti-inflammatory action of plasma acetylsalicylic acid are few because, until recently, no methods have been available for the direct measurement of ASA in body fluids. This study will attempt to develop a technique for direct plasma ASA measurements and to correlate the levels with clinical responses.

Methods Employed:

Previous measurements of plasma ASA levels have been calculated as the difference in the salicylic acid concentrations before and after hydrolysis of an aqueous extract of the sample. Large errors may be present when salicylic acid is present in large excess of ASA. In order to eliminate such errors, direct measurement of ASA is desirable. Spectrophotofluorometry and gas liquid chromotography have been employed for direct determinations of ASA. These techniques will be modified and used for direct determinations of ASA.

Major Findings:

None

Significance to Cancer Research:

Inflammatory reactions result directly and indirectly from tumor cell proliferation. The present study is intended to study drugs which may influence such an inflammatory process.

Proposed Course of Project:

Continuation along lines as outlined.

Honors and Awards: None

Publications: None

Annual Report of the Endocrinology Branch

National Cancer Institute

July 1, 1967 through June 30, 1968

In the broadest sense the Endocrinology Branch is engaged in the study of regulation of tissue growth and differentiation at many levels of organization. At the level of molecular biology, the site and mechanism of steroid hormone action is being investigated. At the cellular level, cytologic and biochemical differentiation in tissue culture in response to hormonal stimuli promise to be fruitful areas for study. In man, analysis of hormone production, secretion, and metabolism is important to define the control of the internal milieu.

In pursuit of these goals, a variety of skills, techniques, and interests are necessary. Within the Branch, therefore, are investigators working in steroid metabolism, protein hormone chemistry and metabolism, nucleic acids, tissue culture, and clinical investigation. Active collaboration among members of the Branch and with other Branches adds greatly to the research potential.

In the molecular biology area, the intensive examination of nuclear RNA in the chick oviduct system has been rewarding. The stimulation of avidin synthesis by progesterone has been localized at the transcription level. The changes in type and base composition of rapidly labeled nuclear RNA have been carefully analyzed during differentiation. The processes of cytologic and biochemical differentiation were examined and the cytologic changes compared with the process of induction of specific protein synthesis. This model system affords an excellent opportunity to examine the interplay of estrogen and progesterone in estrogen-responsive tissue. That these studies are apposite to human cancer is apparent from the comparable effects of these hormones on such tissues as uterine cervix and endometrium and breast.

The initial separation of human prolactin from human growth hormone has been accomplished. This will ensure the future development of methods for prolactin assay. Investigations into the control of the hormonal milieu in man have been facilitated by the development of methods for the analysis of follicle-stimulating hormone, progesterone, and hydroxyprogesterone. The relationship of pituitary gonadotrophin secretion to

follicle growth and steroid secretion in women has been clarified. The results of these studies are not only of interest in endocrine cancers but have considerable importance to several areas of reproductive biology.

The efforts of the Branch have been strengthened by several guest workers and a Visiting Scientist. In addition, the Branch and individual investigators have been hosts to many visitors from this country and throughout the world. We have welcomed the collaboration with members of the National Institutes of Health staff since it has offered an opportunity to enlarge our interests and utilize research projects more profitably. We look forward to another exciting year of research in the many problems of hormonal regulation.

Serial No. NCI-803
1. Endocrinology Branch
2. Bethesda, Md.

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Nature of Hormone-Producing and Hormone-Sensitive Tumors in Man, Including Tumors of Breast, Prostate, Uterus, Pituitary Adrenal, Ovary and Testis; Abnormalities of Somatic Development and Growth.

Previous Serial Number: Same

Principal Investigator: Dr. Mortimer B. Lipsett

Other Investigators: Dr. G. T. Ross, Dr. M. A. Kirschner, Dr. C. Cargille, Dr. H. Cannan, Dr. A. Chrambach, Dr. T. Yoshimi, Dr. C. W. Bardin, Dr. B. O'Malley, Dr. P. A. Kohler, Dr. W. McGuire, Dr. W. C. Hembree, Mr. P. Rayford, Mr. D. W. Ryan, Mr. J. Brice, Miss J. Taylor, Mrs. M. Wyckoff, Dr. C. A. Strott, Guest Worker, Mrs. Pat Middleton.

Cooperating Units: Laboratory of Biology, NCI; Laboratory of Pathology, NCI; Chemistry Branch, NCI; Surgery Branch, NCI; Neurosurgery Branch, NINDB; Laboratory of Biology, NICHD.

Man Years:

Total:	30
Professional:	19
Other:	11

Project Description:

Objectives: 1. To understand mechanisms of hormonal regulation of normal and abnormal tissue growth.

2. To assess the hormonal and other factors that modulate the growth of several endocrine-sensitive cancers, such as carcinoma of the breast and prostate.

Objectives: (cont)

3. To define the regulatory processes involved in maintenance of blood and tissue hormone levels.

4. To attempt to modify hormonal and other factors that modulate the growth of several endocrine-sensitive cancers and thereby palliate the course of the cancer.

Methods Employed: These vary widely since the research encompasses such divergent areas as protein chemistry, molecular biology, steroid analysis, and clinical investigation. The investigators are eclectic in their choice of methods; we have adapted or modified known methods, when possible, and new ones have been developed for special needs.

Hormone Action: - The techniques of special interest are: - measurement of template activity in stripped chromatin, DNA-RNA hybridization, nearest neighbor dinucleotide analysis, enzymatic acylation to estimate tRNA activity, in vitro measurement of mRNA activity, and characterization of nuclear RNA by sucrose and cesium chloride density gradient and polyacrylamide gel electrophoresis.

Regulation: - Techniques useful at this level of cellular organization are tissue culture of human endocrine glands, chick oviduct, and hydrocarbon-induced mammary cancers. Study of these systems is performed utilizing fluorescent antibody staining, electron microscopy, and radioautography. The products of cells in tissue culture have been measured by radioimmunoassay techniques described below.

Protein Chemistry: - This laboratory utilizes standard column chromatography techniques for large-scale preliminary purifications. Analytic and preparative polyacrylamide gel electrophoresis, the use of stacking gels, and multiphasic buffer systems provide sophisticated methods of separation.

Hormone Analysis:- Radioimmunoassays are performed routinely for human growth hormone, thyrotropin, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Steroid hormones are measured in blood or urine by double isotope derivative techniques, electron capture gas-liquid chromatography, and competitive protein-binding. Bioassays for long-acting thyroid stimulator, FSH, LH, and total gonadotropins continue to be necessary.

Major Findings:

A. Hormone Action

The present objectives of this effort are: 1) to elucidate mechanisms of action of the steroid hormones, and 2) to define the sequence of events necessary for transfer of genetic information during growth and differentiation. It was reported last year that progesterone induced the synthesis of a single protein, avidin, by the chick oviduct, in vivo, in minces in vitro, and in monolayer culture. Using this system, the finding that progesterone stimulated RNA synthesis and RNA polymerase activity suggested a primary effect at the transcription level.

Since then, additional studies have strengthened this hypothesis and extended the information regarding other possible loci of action. It was shown that progesterone, administered, in vivo, led to increased template activity of isolated chromatin before the time when avidin synthesis could be measured.

DNA-RNA hybridization techniques were then used to examine mRNA during estrogen-induced tissue growth and progesterone induced avidin synthesis. The amount of non-hybridizable nuclear RNA increased progressively during estrogen-induced tissue growth. When progesterone was given, new non-hybridizable nuclear RNA was found prior to demonstrable avidin synthesis. Thus, induction by a hormone of synthesis of a single protein caused the appearance of a new species of nuclear RNA (mRNA?).

The RNA synthesized in vitro from isolated chromatin was examined by nearest neighbor dinucleotide analysis. There were progressive shifts in the base-pair ratios following progesterone. This study and the previous one constitute strong evidence that one steroid hormone, progesterone, exerts an early and important effect on transcription.

In collaboration with Dr. W. Dingman, Laboratory of Chemistry, NCI, the pattern of nuclear RNA was examined by polyacrylamide-agarose gel electrophoresis. Estrogen produced increases in all fractions but 4S RNA (transfer RNA) was increased many times in relation to other nuclear RNA fractions. This presumed increase of tRNA was confirmed

Major Findings (cont):

by showing subsequently that tRNA activity increased pari passu as measured by enzymatic acylation.

B. Regulation

Differentiated cells of human and several animal tissues have been maintained in long-term monolayer culture. This system has been utilized to study hormone biosynthesis by human pituitary glands and differentiation by combined histologic and biochemical techniques. Human chromophobe adenomas maintained in continuous culture exhibited continuing production of low amounts of thyrotropin and LH as defined by the radioimmunoassay. In view of the commonly held belief that these tumors lack functional activity, these findings suggest that biosynthetic capacities are present and thereby may lead to definition of the origin of these tumors from a discrete cell population. Similar studies of human eosinophilic adenomas obtained from patients with active acromegaly showed that these tumors in tissue culture maintained production of growth hormone, LH, and thyrotropin. Normal pituitary tissue does not long maintain its functional capacity in tissue culture. However, during the period of function, it has been used as a test system to examine the activity of putative hypothalamic releasing factors.

The chick oviduct continues to be a useful tool for studying differentiation. In a collaborative study with Dr. P. M. Grimley, Laboratory of Pathology, NCI, the morphologic concomitants of estrogen-induced differentiation have been examined by light and electron microscopy. The growth of the tissue following estrogen is accompanied by ribosomal aggregation and formation of new endoplasmic reticulum. The low cuboidal undifferentiated epithelium differentiates into three cell types, goblet cells, columnar cells, and ciliated cells. Within the columnar cells large drops of proteinaceous material appear.

Using fluorescent antibody techniques with antibodies prepared against ovalbumin and avidin, the columnar cells were shown to contain ovalbumin but no avidin could be demonstrated in the oviduct. When progesterone was given, fibrillar granules appeared in the goblet cells and the anti-avidin antibody localized to these cells. This location of avidin was confirmed by demonstrating binding of tritiated biotin to these cells by autoradiography. Thus, estrogen induced growth and cytologic

Major Findings: (cont)

differentiation of the oviduct epithelium and progesterone induced biochemical differentiation in one cell population.

C. Protein Chemistry

Methodology continues to occupy some of the resources of the laboratory and other programs. In collaboration with Dr. T. Jovin, Stanford University, a computer program for the calculation of multiphasic buffer systems over wide pH ranges has been developed and tested. All possible discontinuous buffer systems between pH 2.5 and 11.0, using 43 constituent ions (all known suitable ions), both in the cathodic and anodic direction of migration, at 0°C and at 25° C, for at least single sets of input parameters were generated. Selection of model systems for the elaboration of polymerization conditions, experimental verification of physical characteristics predicted by the theory upon which the program is based, and model fractionations of proteins are in progress. The main benefit of multiphasic buffer systems is the auto-concentration of sample prior to fractionation. The availability of these buffer systems will therefore open up for fractionation work, systems frequently encountered in biology, in which the macromolecule of interest occurs in low concentration (less than 0.1 mg/ml). Presently, such buffer systems exist only at the extremes of pH where electrophoretic separation is minimal and many biological activities do not survive.

During the last year several reports appeared in the literature indicating oxidative damage to proteins during polyacrylamide gel electrophoresis caused allegedly by the polymerization initiator, persulfate. Since such damage would undermine the usefulness of polyacrylamide gel fractionations and as the proposition seemed impossible both from the electrophoretic and the thermodynamic viewpoints, it was decided to investigate critically this phenomenon. In collaboration with Dr. M. Dirksen, the Johns Hopkins University School of Medicine, we showed that polyacrylamide gels can form in the presence of an excess reducing agent and that the protein pattern is not dependent on oxidative conditions. It is however sharply dependent upon residual acrylamide monomer in the gel. The nature of the interaction between protein and acrylamide was found to involve the alpha-amino group. The reaction has since been published in the literature. Investigations are in progress to quantify the residual monomer in polyacrylamide by permanganate titration and

Major Findings: (cont)

C. Protein Chemistry (cont)

thereafter to find polymerization conditions that drive polymerization to completion. Thus, although this investigation revealed that the residual persulfate did not damage the proteins, it has opened up basic new problems in polyacrylamide gel electrophoresis.

The concentration of protein solutions following preparative polyacrylamide gel electrophoresis has been difficult because of adsorption of protein during the dialysis. An electrophoretic concentration apparatus has been devised to concentrate solutions containing 0.2 to 1.0 mg of protein per 20 ml. Recoveries are 60% at present and will be improved by further modifications.

In collaboration with Dr. M. Ben-David, Laboratory of Biology, NCI, detailed study of prolactin was undertaken. Before approaching the more difficult problem in the human, rat prolactin was examined. Contrary to earlier reports, the biologically active protein was not visibly stained in electrophoretic patterns of rat pituitaries at pH' = 9.45 (25°C) or 7.8 (0°C). However, the pattern of pituitary extracts prepared by adaptation of the percolation procedure of Bates yielded a strongly active band of R_f 0.58 (pH' = 7.8, 10% acrylamide). This band was also demonstrated in the serum of lactating and perphenazine-treated rats. Thus, the natural prolactin and the lactogenic activity induced by perphenazine appear to be identical.

The bioassay procedure necessary to support these studies was facilitated by utilizing electrophoresis to move the active material from the gel slices into agar. The agar could then be used directly in the crop-sac assay.

When rat serum was extracted, the active band could not be seen because of the many protein bands present. However, after electrophoresis of multiple gel slices into a concentration gel and of the stack into 0.2% agar, prolactin activity was demonstrated. A protein band was isolated with the same R_f as that of pituitary prolactin. Thus, the feasibility of this approach for concentrating and isolating prolactin activity from normal rat serum was shown.

Major Findings: (cont)

C. Protein Chemistry (cont)

Using partially fractionated human pituitary preparations, three prolactin bands have been demonstrated. These were not seen in the best preparations of human growth hormone. This is the first direct demonstration that human prolactin is different from growth hormone. Activity ratios and specific activities are being examined.

The laboratory has continued to serve as a focal point for investigators both within and outside the Branch who have problems demanding protein separation. A few examples of these collaborative efforts are: 1) The growth hormone synthesized by acromegalic pituitaries in tissue culture was shown to be identical with human pituitary growth hormone; 2) The steroid-binding protein that binds estrogen and testosterone was located on the electrophoretic map of serum proteins. Studies indicate that the protein can be isolated by selective stacking in preparative gel electrophoresis; 3) Studies in isolation of protein mucopolysaccharide complexes were begun; 4) Fractionation of RNA by gel electrophoresis was improved; 5) Fractionation of human chorionic gonadotropin was undertaken and systems devised for its isolation.

D. Clinical Investigation

The development of a precise accurate radioimmunoassay for plasma FSH has facilitated studies of the regulation of ovarian function. The levels of FSH and LH have been measured daily in 21 normal young women. The presence of an early FSH peak preceding the LH peak was noted. The response of FSH and LH to clomiphene citrate provided a basis for evaluating the integrity of the hypothalamic pituitary axis. For the first time measurements of the blood production rates of FSH and LH were made in normal women, in women on contraceptive agents, and in postmenopausal women.

The availability of gonadotropin and androgen methods facilitated the analysis of hypogonadism. The syndrome of hypogonadotropic hypogonadism has been defined and was shown, for the first time, to include several associated defects such as skeletal anomalies , hyposmia and Leydig cell unresponsiveness.

Major Findings: (cont)

D. Clinical Investigation (Cont)

Urinary excretion of FSH and LH were studied by radioimmunoassay and bioassay of aliquots of concentrates of pools of urine collected from prepubertal children. The amounts of FSH and LH and their ratios were determined and compared with the levels in urine of normal adult men. The urinary concentration of FSH and LH per liter of urine of prepubertal children were approximately one-half to one-tenth of the concentration of those same hormones in adult males. The ratios of FSH:LH were therefore of the order of magnitude of 10 in the urine of prepubertal children as compared to a ratio of 1 in normal adult males suggesting that pubescence is accomplished by a relatively greater increase in urinary excretion of LH than of FSH.

In collaboration with Dr. John Marshall, Surgery Branch, NCI, plasma chorionic gonadotropin levels have been studied in early pregnancy among women who conceived following ovulation induced by administration of postmenopausal urinary gonadotropin followed by HCG. Results showed that from day 12 to 21 after ovulation plasma HCG levels increased exponentially with a doubling time of 1.7 days. This rate of increase may reflect the rate of increase in numbers of hormone producing cells during the same period.

There has been a continuing interest in the problems of human growth. Arginine, insulin, and pyrogen were used to test growth hormone responsiveness and it was shown that no single test is sufficient to predict accurately the capacity to release growth hormone.

The studies of androgen physiology have been expanded to cover a variety of virilizing syndromes. It has now been proven that the adrenal cortex is an important source of blood testosterone in the polycystic ovary syndrome.

Detailed examination of the role of androgen secretion in the maintenance of plasma estrogens is under way. The fractional conversion rates from testosterone to estradiol and androstenedione to estrone were 0.0045 and 0.012. From the calculated production rates, it was estimated that, in men, testosterone secretion was the major source of plasma estradiol. The necessity for achieving equilibrium conditions before determining fractional conversion rates was emphasized by the finding that

D. Clinical Investigation (cont)

these rates for interconversion of the estrogens continue to increase during 6 hours of infusion.

Competitive protein-binding methods were devised to measure plasma progesterone and, for the first time, plasma 17-hydroxyprogesterone. It was shown that plasma progesterone in women did not increase until after the LH peak. Plasma 17-hydroxyprogesterone levels started to rise before the LH peak and decreased with progesterone before the onset of menses. The data suggest that the activity of two cell populations was being measured; progesterone produced by luteinized cells and 17-hydroxyprogesterone from persistent theca interna cells.

In congenital adrenal hyperplasia, 17-hydroxyprogesterone levels were elevated 100 to 200 times and production rates were 50 to 120 mg per day. This steroid was also shown to be a good indicator of Leydig cell function since these cells produce about 2 mg of 17-hydroxyprogesterone daily.

Electron capture gas-liquid chromatography has been utilized to simplify the determination of plasma testosterone and androstenedione. New derivatives have been made to increase the sensitivity of the method so that it will be applicable for plasma estrogens.

Men with metastatic choriocarcinoma have been studied and treated. Curiously, in spite of high levels of chorionic gonadotropin, plasma testosterone levels are not elevated. We have shown that there is little response to exogenous HCG as well. This raises the possibilities of either antibodies to tumor HCG or secretion of a biologically inactive HCG by the tumor.

In collaboration with Dr. Louis Alpert, George Washington University School of Medicine, we are attempting to perform medical adrenalectomy in patients with metastatic breast cancer. Aminoglutethemide, a drug that blocks steroid biosynthesis in the adrenal cortex is given with small doses of prednisone to prevent increasing ACTH levels. This study has just been started but it seems likely from few preliminary data that complete suppression of adrenocortical function will be possible.

Significance to Institute Program and Bio-Medical Research:

Endocrine therapy of such cancers as carcinoma of the breast, prostate, and uterus is largely empiric. The possible predictive and diagnostic aspects of hormonal analyses in these diseases has been demonstrated only recently. It is apparent that much of the work over the past fifteen years was not valid because methods of analysis were inadequate and appreciation of the complexities of hormone secretion was insufficient. A major effort of this Branch has been the development of methods for the analysis of the circulating protein and steroid hormones. This has enabled us to investigate production, metabolism, and regulation of these hormones. These efforts provide the necessary background to the meaningful study of the relationships of hormones with endocrine-responsive tumors.

Hormonal control of tissue growth is one aspect of the general problem of regulation of normal and abnormal cell growth. The development of model systems for studying hormonal regulation is a first important step in this area. Results obtained in this system will provide significant direction to many probing studies in hormonal regulation.

The development of several methods by the Branch has permitted research in fields not possible previously. In cancer endocrinology, reproductive biology, and basic endocrinology, specific research programs have been facilitated because of this. Thus, the studies within the Endocrinology Branch have been significant to the community of endocrinology and internal medicine.

Proposed Course:

The following research efforts will be given priority during the coming year:

1) Development of a soluble system for studying hormonal induction of protein synthesis.

2) The adaptation and use of tissue culture methods for the study of protein hormone synthesis and regulation.

Proposed Course: (cont)

3) The study of hydrocarbon-induced mammary cancer in tissue culture with respect to changes in nuclear RNA metabolism.

4) Measurement of plasma estrogens and their origin particularly in the post-menopausal patient.

5) The development of a radioimmunoassay for prolactin and the subsequent investigation of its normal physiologic role and its possible participation in breast cancer growth.

6) The continuing study of pituitary-gonadal physiology in health and in specific diseases.

7) The further clinical studies in and treatment of patients with metastatic breast cancer and testicular cancer.

Honors and Awards:

Dr. Mortimer B. Lipsett

Invited Lectures:

Gas Chromatography Symposium, Chicago, Nov. 15, 16, 1967.

Endocrine Conference, University of Maryland, Nov. 20, 1967.

Howard University School of Medicine, Dec. 1, 10, 1967.

George Washington University Hospital, January 17, 1968.

Walter Reed Hospital, Washington, D. C., Feb. 12, 1968.

Schering Symposium, Berlin, March 14, 1968.

University of Cambridge, Cambridge, March 19, 1968.

Panelist & Lecturer - Southeastern Surgical Congress, March 26, 27, 1968.

Appointed Editor-In-Chief, Journal of Clinical Endocrinology and Metabolism, Jan. 1968.

Appointed to Reproductive Biology Study Section, June 1968.

Dr. Griff T. Ross

Chairman, Clinical Research Committee of the Medical Board (re-elected)

Member-at-Large - Medical Board (re-elected)

Associate Editor - The Journal of the National Cancer Institute (reappointed)

Member - Clinical Trials Contract Review Committee (re-elected)

Honors and Awards: (cont)

Dr. Griff T. Ross (cont)

Member - NCI Committee on Diagnostic Research (re-elected)

Appointed Associate Editor - Journal of Clinical Endocrinology and Metabolism

Member - National Pituitary Agency Gonadotropin Subcommittee

Member - Choriocarcinoma Cooperative Study

Invited Lectures:

Blackford Memorial Lectures, Sherman, Texas, Sept. 10, 1967.

Endocrine Conference at Columbia University College of Physicians and Surgeons, New York, N. Y., Oct. 13, 1967.

Fourth Rochester Trophoblastic Conference, Rochester, N.Y., Oct. 30-31, 1967.

Workshop Conference on Statistics in Endocrinology, Dedham, Mass., Dec. 4-8, 1967.

Massachusetts Institute of Technology seminar, Cambridge, Mass., Jan. 12, 1968.

Dr. Marvin A. Kirschner

Appointed Asst. Prof. of Medicine, Georgetown University School of Medicine, Sept. 1967.

Honors and Awards: (cont)

Dr. Marvin A. Kirschner (cont)

Invited Lectures:

D. C. General Hospital, Washington, D. C., Nov. 1967.

Albert Einstein College of Medicine, New York, Feb. 1968.

Dr. C. Wayne Bardin

Invited Lectures:

George Washington School of Medicine, March 1968.

Cornell University School of Medicine, May 1968.

Dr. Bert W. O'Malley

Invited Lectures:

Duke University School of Medicine, Nov. 1967.

Kettering Research Institute, Yellow Springs, Ohio, Dec. 1967.

Dr. Peter O. Kohler

Invited Lectures:

Therapeutic Research Seminar, Baltimore, Md., Oct. 17, 1967.

Publications:

Clinical Investigation:

Bardin, C. W., and Lipsett, M. B.: Testosterone and androstenedione production and interconversion rates in hirsute women. In Tamm, J. (Ed.): Proc. Workshop Conf. on Testosterone, Tremsbüttel, Germany, April 1967. Stuttgart, George Thieme, 1968, pp. 226-231.

Bardin, C. W., Lipsett, M. B., Edgcomb, J. H., and Marshall, J. R.: Studies of testosterone metabolism in a patient with masculinization due to stromal hyperthecosis. New Eng. J. Med. 277: 399-402, Aug. 24, 1967.

Bardin, C. W., Lipsett, M. B., and French, A.: Testosterone and androstenedione production rates in patients with metastatic adrenal cortical carcinoma. J. Clin. Endocr. 28: 215-220, Feb. 1968.

Bardin, C. W., Ross, G. T., and Lipsett, M. B.: Site of action of clomiphene citrate in men: a study of the pituitary-Leydig cell axis. J. Clin. Endocr. 27: 1558-1564, Nov. 1967.

Kimball, H. R., Lipsett, M. B., Odell, W., and Wolff, S. M.: Comparison of the effect of the pyrogens, etiocholanolone and bacterial endotoxin on plasma cortisol and growth hormone in man. J. Clin. Endocr. 28: 337-342, March 1968.

Kohler, P. O., Phang, J. M., Tullner, W. W., Ross, G. T., and Odell, W. D.: Effect of iodination on the biological activity and metabolism of human luteinizing hormone. J. Clin. Endocr. In press for publication May 1968.

Kohler, P. O., Ross, G. T., and Odell, W. D.: Metabolic clearance and production rates of human luteinizing hormone in pre- and postmenopausal women. J. Clin. Invest. 47: 38-47, Jan. 1968.

Yoshimi, T., and Lipsett, M. B.: The measurement of plasma progesterone. Steroids 11: 527-540, April 1968.

Publications: (cont)

Clinical Investigation (cont)

Kulin, H. E., Kohler, P. O., O'Malley, B. W. and Odell, W. D.: Thyroid-stimulating hormone in thyroid dysgenesis. J. Pediat. 71: 714-717, Nov. 1967.

Kulin, H. E., Rifkind, A. B., and Ross, G. T.: Bioassay determinations of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in adult male plasma. J. Clin. Endocr. 28: 100-102, Jan. 1968.

Lipsett, M. B., and Bardin, C. W.: Double isotope derivative measurement of plasma testosterone and androstenedione. In Tamm, J. (Ed.): Proc. Workshop Conf. on Testosterone, Tremsbüttel, Germany, April 1967. Stuttgart, George Thieme, 1968, pp. 9-12.

Lipsett, M. B., Migeon, C. J., Kirschner, M. A., and Bardin, C. W.: Physiologic basis of disorders of androgen metabolism. Ann. Intern. Med., in press for publication June 1968.

Odell, W. D., Hertz, R., Lipsett, M. B., Ross, G. T., and Hammond, C. B.: Endocrine Aspects of Trophoblastic Neoplasms. In Goldstein, D. P., and Gore, H. (Eds.): Symposium on Trophoblastic Disease. New York, Hoeber Medical Division, Harper & Row, vol. 10, June 1967, pp. 290-302.

Odell, W. D., Rayford, P., and Ross, G. T.: Simplified, partially automated method for radioimmunoassay of human thyroid stimulating hormone, growth, luteinizing, and follicle stimulating hormones. J. Lab. Clin. Med. 70: 973-980, Dec. 1967.

Rifkind, A. B., Kulin, H. E., and Ross, G. T.: Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the urine of prepubertal children. J. Clin. Invest. 46: 1925-1931, Dec. 1967.

Ross, G. T., Hammond, C. B., and Odell, W. D.: Chemotherapy for nonmetastatic gestational trophoblastic neoplasm. Clin. Obstet. Gynec. 10: 323-329, June 1967.

Publications: (cont)

Clinical Investigation (cont)

Wurtman, R. J., Axelrod, J., Vesell, E. S., and Ross, G. T.: Species differences in inducibility in Phenylethanolamine-N-Methyl transferase. Endocrinology 82: 584-590, March 1968.

Hormone Action:

Korenman, S. G., and O'Malley, B. W.: Avidin assay: a new procedure suitable for tissue fractions. Biochim. Biophys. Acta 140: 174-176, 1967.

Korenman, S. G., and O'Malley, B. W.: Hormonal regulation of specific protein synthesis: control of avidin induction in chick oviduct by estrogen and progesterone. Endocrinology, in press for publication May 1968.

McGuire, W. L., and O'Malley, B. W.: Ribonucleic acid polymerase activity of the chick oviduct during steroid-induced synthesis of a specific protein. Biochim. Biophys. Acta 157: 187-194, March 18, 1968.

O'Malley, B. W.: In vitro hormonal induction of a specific protein (avidin) in chick oviduct. Biochem. 6: 2546-2551, Aug. 1967.

O'Malley, B. W., Kirschner, M. A., and Bardin, C. W.: Estimation of plasma androgenic and progestational steroids in the laying hen. Proc. Soc. Exptl. Biol. Med. 127: 521-523, Feb. 1968.

O'Malley, B. W., and Kohler, P. O.: Hormonal induction of specific proteins in chick oviduct cell cultures. Biochem. Biophys. Res. Commun. 28: 1-7, 1967.

O'Malley, B. W., and Korenman, S. G.: Studies on the mechanism of hormone induction of a specific protein. Immunological identity and kinetic studies of avidin synthesized in vitro by the chick oviduct. Life Sci. 6: 1953-1959, 1967.

O'Malley, B. W., McGuire, W. L., and Korenman, S. G.: Estrogen stimulation of synthesis of specific proteins and RNA polymerase activity in the immature chick oviduct. Biochim. Biophys. Acta 145: 204-207, 1967.

Publications: (cont)

Hormone Action (cont)

O'Malley, B. W., McGuire, W. L., and Middleton, P. A.: Structure-function relationships of various steroids relative to induction of a specific oviduct protein (avidin). Endocrinology 81: 677-678, Sept. 1967.

Regulation:

Kohler, P. O., Grimley, P. M., and O'Malley, B. W.: Protein synthesis: differential stimulation of cell-specific proteins in epithelial cells of the chick oviduct. Science 160: 86-88, 1968.

Kohler, P. O., and O'Malley, B. W.: Estrogen-induced morphologic changes in monolayer cultures of immature chick oviduct. Endocrinology 81: 1422-1427, Dec. 1967.

O'Malley, B. W., and Kohler, P. O.: Studies on steroid regulation of synthesis of a specific oviduct protein in a new monolayer culture system. Proc. Natl. Acad. Sci. 58: 2359-2366, Dec. 1967.

O'Malley, B. W., and McGuire, W. L.: Studies on the mechanism of action of progesterone in regulation of the synthesis of specific protein. J. Clin. Invest. 47: 654-664, March 1968.

SUMMARY

Annual Report of the Immunology Branch, National Cancer Institute

July 1, 1967 through June 30, 1968

I. Antibody Molecules and Immunoglobulin Genes.

The combination of studies being conducted in the Immunology Branch provide new information about antibody molecules which is equaled in very few laboratories of the world. This far ranging approach also provides rapid evaluation of theories about the genetic mechanisms for generating antibody diversity.

An example of this is the Oz story which began a year ago in the Immunology Branch with the finding of two types of human lambda (light) polypeptide chains. These differed in having either arginine or lysine at amino acid position #190 in the sequence of 212 amino acids. One obvious interpretation would be that these two forms represent the product of two allelic genes (such as the genes determining normal and sickle hemoglobin which also differ only by a single amino acid substitution in their corresponding polypeptide chains). Further studies by D. Ein, however, show that both Oz groups are present in all individuals studied. Thus, allelic genes are ruled out as a cause for the two Oz groups, and the interpretation is now somewhat more interesting but unresolved. The phenomena could be caused by two different genes (coexisting in all individuals) which code for two different lambda chains. Alternatively, ambiguity in the system for translating a single structural gene into the final polypeptide chain could also produce the two forms noted. Further studies of this problem will certainly be interesting.

The antibody molecule is composed of heavy as well as light polypeptide chains. Studies of heavy chains have started, but the light chains of both kappa and lambda types have been more intensively investigated in the past year. Both chains are composed of two regions, each roughly half of the chain, which appear to vary independently. If the variations in each half can be shown to be under separate gene control, this would upset the prevailing notion of molecular biology that one gene determines one polypeptide chain and would help account for antibody diversity (and specificity) which are fundamental features of the immune system.

Prior work has shown that the common or constant region (half) of kappa chains is controlled by a single gene. L. Hood has shown by amino acid sequence studies that the specificity (or variable) region (half) may occur in at least three forms or subclasses. These findings indicate that this part of each kappa chain is determined by one of at least three, and probably more, germ-line genes. A similar situation exists for lambda chains which have been studied in great detail by Drs. Hood and Ein. In both situations, three is the minimal number and many more genes may well control the specificity (or variable) half of the molecule. If this is the case, it seems

likely that these genes provide (or contribute to) antibody specificity.

Actually, the polypeptide chains which have been studied are not from proven antibody molecules but are obtained from Bence Jones proteins and myeloma proteins. These products of malignant cells provide essential material and unique opportunities to investigate the immunoglobulin system.

Studies of conformational antigens, i.e. those requiring both heavy and light chains, were done with IgM molecules by W. Terry and E. Miller. They showed that both mu (heavy) and kappa (light) polypeptide chains were necessary for expression of one specific conformational antigen. The significance of this finding will only be understood when they can be related to three-dimensional structural work of a type that is being undertaken now by W. Terry.

II. Clinical and Diagnostic Studies with Immunoglobulins.

A unique disease was found by W. Terry (Immunology) and T. Waldmann (Metabolism). Studies of patients with various immunoglobulin disorders led them to the identification of a brother and sister who have increased catabolism of IgG and albumin. No similar patients have been reported previously nor has any comparable animal disorder been described. The nature of this defect is not yet clear, and the studies of the turnover of other serum proteins are being pursued. Albumin and IgG synthesis is normal in these patients. The disorder seems to be entirely in the area of increased catabolism.

A patient with the rare "Heavy-Chain Disease" was identified. Previously it was not certain whether the anomalous protein that accumulates in these patients was synthesized as a heavy-chain piece or resulted from unusual catabolism (digestion) of normal IgG molecules. Biosynthetic and immunofluorescent studies in the Immunology Branch showed that the cells in the patient's bone marrow synthesized the heavy-chain piece without also forming light-chains in the same cells. Future studies will focus on the biosynthetic defect in these cells.

The NCI Immunoglobulin Reference Center continued to supply service and assistance to the research community investigating human and mouse immunoglobulins. Over a thousand serums were tested and 455 were found to contain abnormal serum proteins. More important than the number of anomalous immunoglobulins identified was the assistance given to many investigators who recognized the presence of anomalous immunoglobulins but were unable to characterize or identify them. Of even greater importance, perhaps, were the reagents sent to 68 investigators. Over 150 lots of reagents were supplied for identification of specific human immunoglobulins.

In addition, assistance was provided to investigators studying the immune system of mice. Murine plasma cell tumors that synthesize immunoglobulins were sent to 16 institutions. Eighteen investigators received reagents for identifying 5 specific mouse immunoglobulins.

The NCI Immunoglobulin Reference Center is leading a project to establish a National (and International) Reference Serum which could be used as a standard for quantification of human immunoglobulins. A stable preparation has been obtained. A collaborative effort to calibrate the Reference Serum is underway. We hope this can be finished and distribution of the Reference Serum can begin in the coming year.

III. Lymphoid Cell Regulation.

Efforts to regulate immunoglobulin formation have taken advantage of the finding originating in this laboratory that immunoglobulin synthesis can occur in human lymphoid cells in culture. The new finding that individual cells in culture can form two kinds of heavy chains and (probably) two kinds of light chains is in striking contrast to studies of normal or malignant plasma cells in vivo where only a single heavy and light chain are formed, i.e. only a single gene for the heavy chain and a single gene for the light chain are expressed in vivo. In these cultures, however, many genes are being expressed. This indicates that special conditions exist in the tissue culture situation that do not normally exist in vivo. This difference is being explored in an effort to identify regulatory mechanisms for immunoglobulin gene expression and antibody formation.

Production of specific antibody on a large scale for clinical use in man is one goal of work in this laboratory. Although numerous attempts to induce antibody formation in the human lymphoid tissue cultures have failed, we are endeavoring to use histocytes, macrophages and similar cells and their products to direct synthesis in the direction of specific antibody. Also, we are attempting to establish cultures of cells which already have the capacity to produce antibody.

In the past year we have become persuaded that the human lymphoid cells growing in culture may not represent malignant cells at all, even though they are obtained from Burkitt tumor patients or leukemia patients. Although these are often referred to as Burkitt lines or leukemia lines there is no positive proof that these cells are malignant or represent malignant cells. Indeed, there is a good bit of indirect evidence indicating that they are not the typical malignant cell of the tumor. Whether these cultures are an outgrowth of normal cells or represent stem cells of the tumor remains to be determined. This problem is leading into questions of the nature of neoplastic cell, the type of cell that will grow in culture and the comparison of neoplastic and normal cells in vivo. Immunoglobulin production is a handle which enables us to tell something about gene expression in these lymphoid cell cultures. Studies have already begun on the isoantigen composition of these cells (see Rogentine) and will be extended to obtain more information about the membrane structure of the various forms of malignant, normal and tissue culture cells.

IV. Transplantation Immunology.

Transplantation research is developing in several directions within the Immunology Branch:

1) Preparation of soluble histocompatibility antigens for clinical and molecular biology studies

2) Human histocompatibility (HLA) typing systems

3) Enhancing antibodies

4) Immune suppression with anti-lymphocyte serum and chemotherapy.

The clinical applications of transplantation research in the Immunology Branch are directed toward bone marrow and other hematopoietic cell transfer. A clinical program has been prepared and candidates for bone marrow transplantation are being admitted.

Laboratory work with transplantation of bone marrow has been developed with inbred mouse systems. Furthermore, negotiations are underway to obtain a primate facility in which bone marrow transplantation in monkeys and possibly other larger primates can be carried out. It is hoped to begin this program within the next few months. The need for mouse and primate systems is indicated by the complexity of the clinical situations in which bone marrow grafts or hematopoietic cell grafts are done. and the need for animal systems in which variables can be tested. It seems certain that immune suppression will be a multi-phasic procedure for some time and working out the best modes of immune suppression is going to require extensive work in animal systems.

Very exciting progress was made this year in preparing soluble human (transplantation) alloantigen. These glycoproteins probably have a MW of around 60,000 or 70,000. With further knowledge of the amino acid and carbohydrate composition it should be possible to begin to relate genetic and structural information on these important cell membrane components.

In another phase of this work, the soluble alloantigens will be prepared in quantity for biologic testing in man to see if specific tolerance can be induced. Biologic testing of this hypothesis has begun already in mouse systems and will be extended to primate systems and, as soon as feasible, to patients.

Human histocompatibility (HL-A) typing facilities are established in the Immunology Branch by Dr. Rogentine who also participated in the Histocompatibility Typing Workshop in Torino, Italy. Typing has been used to support transplant studies in the Medicine Branch, NCI, and other institutions in the Washington and Baltimore area.

Heterologous anti-human lymphocyte serum has been prepared and immune globulin will be obtained. Heterologous anti-lymphocyte antibodies, homologous isoantiserum, soluble antigens, drug and other technics for immunosuppression will be utilized to facilitate graft take and proper function.

V. Tumor Immunology.

Two lines of work are developing:

1) Efforts to develop assays to detect tumor specific antigens so that purification and a better understanding of the nature of these substances can be obtained.

2) Attempts to take advantage of current advances in immunology to design specific immunotherapy for neoplastic disease.

Dr. Herberman extended his tests for cytotoxic activity in human serum to show that this activity is rare in infants but increases until normal adult levels are reached by age 14. The antigen detected in his system is not a leukocyte alloantigen and is not a tumor specific antigen. Dr. Herberman has found the antigen on all human nucleated cells tested so far. It is also present in the nucleated cells of other mammalian species and, indeed, a cross-reacting antigen has been found in bacterial systems. It would appear that the antibody detected in the cytotoxic assay developed by Dr. Herberman is an antibody against an antigen that is widely distributed in nature. The existence of such antigens must be recognized when tests for so-called tumor specific antigens are claimed.

Because of the difficulties of detecting tumor-specific antigens with currently available technics, considerable effort is being made to improve methods and develop more specific and sensitive tests. Radioisotope-labeled antibody techniques and increasingly sensitive cytotoxicity techniques are being developed. These will be explored with histocompatibility systems and with tumor antigen systems using animal models, and, of course, will be used in efforts to detect specific antibody in patients with neoplastic disease.

Antigenic modification of tumors was done in an effort to increase or induce immune response against the tumor. This work was done in animal systems using a plasma cell tumor (LPC1) and a melanoma (S91) which were modified by treatment with bisdiazobenzidine and coupling with foreign proteins such as rabbit gamma globulin or hemocyanin. Extensive work by M. T. McEnany with over two thousand mice demonstrated that modification of tumor cells may cause some increase in immunity. Similar modification of normal spleen cells, however, had the same effect. The reaction shown here should not be confused with specific anti-tumor immunity. On the basis of these extensive animal studies, it is felt that attempts at immunotherapy using this approach in man are not warranted at the present time.

Another approach to the immunotherapy of cancer is based on the hypothesis that neoplastic cells may be more reactive to antibody damage than normal cells and that alloantibodies against antigens of the HLA system may have useful anti-tumor effect. Such an alloantiserum has been given to several recipients both by local injection and by systemic administration to find out if alloantibodies can be used in the treatment of leukemia or lymphoid disorders.

More specific anti-tumor immunity may exist in patients with disease remission. Although laboratory tests may not reveal this immunity, efforts at bioassay detection of such immunity have been initiated. Serum antibody activity is sought through local and systemic administration. Cellular immunity is being tested by intradermal testing of cell membrane components. This is being done in cooperation with other clinical branches of the NCI so that a range of clinical diseases can be investigated. Studies of multiple myeloma and lymphoma have begun already in the Immunology Branch.

To facilitate the preclinical work a laboratory facility has been established at Melpar, Inc., through an existing contract, in an effort to assemble animals and personnel suitable to carry out studies in preclinical model systems.

Immunology of neoplastic disease is complex and multifaceted.The Immunology Branch feels that studies of immunoglobulin structure and genes, studies of the composition and development of cell membrane, the regulation of immunoglobulin production and studies of transplantation immunology, investigation of normal cell antigens and techniques for enhancing or reducing immunity are all relevant to tumor immunology.

Serial No. NCI-5000 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Antibodies and Immunoglobulins: Structure and Function

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M. D.

Other Investigators: Daniel Ein, M. D., Leroy Hood, M. D., Ph. D.

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: The object of this project is to define the immunoglobulin system and to relate the structure and function of immunoglobulin molecules.

Methods Employed: Heterogeneity and composition of the immunoglobulin molecules are defined by physicochemical, immunochemical and other characteristics. Serum and urine are fractionated by electrophoretic, chromatographic and gel filtration technics to obtain purified immunoglobulin components. Polypeptide chains were prepared by chemical reduction and alkylation and other subunits by enzymatic technics. Biologic activity was determined by *in vivo* and *in vitro* tests.

Major Findings: Last year we identified two types of human lambda light chains (Bence Jones proteins) designated Oz (+) and Oz (-) (Ein and Fahey). Further studies by Dr. Ein and Appella showed that single amino acid substitution (Arginine: Lysine) in position 190 distinguished Oz (+) and Oz (-) lambda Bence Jones proteins.

These findings were extended to lambda chains of normal immunoglobulin molecules by immunochemical and protein chemistry technics (Ein). These studies showed that the Oz groups did not represent allelic genes in the Caucasian population ($p < 1:5000$).

Significance to Bio-Medical Research and the Program of the Institute: In spite of the similarity of the Oz groups to the allelic Inv genes of human kappa chains, and to the sickle cell changes in hemoglobin, the evidence is compelling that Oz groups are not allelic in normal individuals. The view that two genes may code for the common region of an immunoglobulin polypeptide chain is a unique finding. The alternative explanation of translational ambiguity at position 190 is equally interesting, and susceptible to testing.

Proposed Course of Project: (1) Genetic studies of lambda immunoglobulins will be extended at both human population and molecular composition levels. (2) Efforts will be made to identify Oz (-) lambda chains by immunochemical technics so that quantitative studies can be done in human populations to determine the relative amounts of these peptide chains in various genetic and disease groups. (3) Studies with immunoglobulin-D (IgD) will be expanded by isolating the delta heavy chains and lambda light chains for investigation of amino acid composition and comparison with related polypeptide chains. (4) Efforts to define the levels of heterogeneity in the immunoglobulin system will be extended, and relationships between structure and function will continue to be sought. (5) New methods to detect small quantities of immunoglobulins will be developed.

Honors and Awards: None

Publications:

Fahey, J. L.: Chromatographic separation of immunoglobulins. Methods in Immunol., in press.

Fahey, J. L., and Terry, E. W.: Ion exchange chromatography and gel filtration. In Weir, D. M. (Ed.): Handbook of Experimental Immunology. Oxford and Edinburgh, Blackwell Scientific Publications, 1967, pp. 19-43.

Ein, D.: Non-allelic behavior of the Oz groups in human lambda immunoglobulin chains. Proc. Natl. Acad. Sci., in press.

Serial No. NCI-5001 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: NCI Immunoglobulin Reference Center

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M. D.

Other Investigators: William D. Terry, M.D., Daniel Ein, M.D.

Cooperating Units: None

Man Years: (computed for the 12-month period)

Total:	1-1/2
Professional:	1/2
Others:	1

Project Description:

Objectives: The long-range objectives of this project are to find and apply serum diagnostic tests for neoplasms in man. The immediate goals are to establish an NCI Immunoglobulin Reference Center in order to (1) improve immunochemical tests for multiple myeloma and macroglobulinemia by increasing the quality and availability of reagents, technics and interpretation experience, (2) to facilitate studies of immunoglobulins by establishing national reference standards and by making available to qualified investigators small amounts of reagents that are not commercially available, and (3) to investigate the relevance of serum immunoglobulin changes to disease prognosis and response to therapy.

Methods Employed: Immunoelectrophoresis, employing a battery of specific antiserums, is used to detect specific immunoglobulin abnormalities. Quantitative immunochemical technics, principally radial diffusion in agar plates containing antibody, are used to measure the serum levels of IgG, IgA, IgM and IgD, as well as type K and type L immunoglobulins. Protein purification procedures (see Project NCI-5000) are used to prepare immunoglobulins for use as antigens to induce potent antiserums and as absorbents to obtain specific antiserums. Antiserums are produced in rabbits and larger animals.

Major Findings: The principal effort on this project is conducted through a contract with Melpar (#PH 43-66-3). The NCI Immunoglobulin Reference Center has been established at Melpar, Inc. in Springfield, Virginia. During the year there has been further increase in the level of competence of the personnel. Two professional persons are now responsible for the work at the NCI Immunoglobulin Reference Center.

Number of serum and urine tested 4/1/67 to 3/31/68	1,070
Number serums with anomalous immunoglobulins	455
Number of serums with significant immunoglobulin deficiencies	25
Number of antisera to human protein supplied to 68 investigators (3 cc each)	504
Number of antisera to mouse protein supplied to 18 investigators (3 cc each)	68
Number of mouse tumors supplied to 16 investigators	44
Number of visitors who were taught techniques or interpretations	4

Large volumes of seven different antiserums are required for the diagnostic and quantitative studies. The Reference Center has immunized both small and large animals, collected the antiserums, evaluated them for strength and specificity and, where necessary, have absorbed them with appropriate sera or purified immunoglobulin preparations to render them useful for the immunodiagnostic work.

Immunoglobulin standards are needed, both at the Reference Center and at other institutions doing quantitative immunoglobulin studies throughout the country. In the past year a large part of plasma was secured and lyophilized in 1 ml aliquots for use as a standard preparation. The stability of this material has been checked at three and six month intervals and found satisfactory. Calibration of this reference material with purified protein preparations has begun. It is hoped that the analytic results and statistical analyses can be completed in the coming year so that this national standard can be established.

Significance to Bio-Medical Research and the Program of the Institute: Clinicians concerned with diagnosis and care of patients and clinical pathologists concerned with laboratory testing have shown great interest in the specific immunochemical (immunoelectrophoretic) tests suitable for screening serum and urine for specific changes characteristic of multiple

myeloma and Waldenström's macroglobulinemia. The tests eventually can be carried out throughout the country, but proper performance is required. The Immunoglobulin Reference Center has begun to provide a focus for materials and technics.

The development of easily performed quantitative tests for immunoglobulins is accelerating research into the effects of disease, chemical therapy and other events on the individual components of the immunoglobulin system. The standardized procedures and high quality reagents at the NCI Immunoglobulin Reference Center provide reference materials and standards for immunoglobulin investigations in the United States.

The serums referred to the NCI Immunoglobulin Reference Center provide a valuable source of material for research conducted in projects on immunoglobulin structure (see Project NCI-5000), subclasses and genetic factors (see Projects NCI-5002 and NCI-5010) and malignant diseases (see Project NCI-5005).

Proposed Course of Project: The NCI Immunoglobulin Reference Center will continue diagnostic work and production of needed reagents.

Efforts to improve reagents by introduction of technics such as adsorption with insoluble immune adsorbents will be pursued.

Reagents to identify a newly recognized immunoglobulin class, IgE, will be obtained and a search for a patient with an E-myeloma protein will be developed. No such patient has been found in the United States.

Standard Reference Serums, containing known amounts of human IgG, IgA, IgM and IgD, will be established in cooperation with major investigators in this country and with equivalent laboratories in Great Britain, Belgium and Switzerland in cooperation with the World Health Organization.

Mice have an immunoglobulin system similar to that in man. Reagents for identifying and quantitating different mouse immunoglobulin groups are needed in this and other laboratories. The procedures required for this work are similar to those used for human systems. A small capacity to produce these reagents has been developed and will be maintained at the NCI Immunoglobulin Reference Center to support immunoglobulin research in inbred mice. Also a reserve of mouse plasma cell tumors will be established to facilitate supplying these tumors to investigators.

Honors and Awards: None

Publications: None

Serial No. NCI-5002 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Genetic, Structural and Functional Characteristics of Human Immunoglobulins

Previous Serial Number: Same

Principal Investigator: William D. Terry, M.D.

Other Investigators: Thomas Waldmann, M.D., Lee Hood, M.D., Ph.D., Edward J. Miller, M.D.

Cooperating Units: Human Genetics Branch, NIDR, Joost Oppenheim, M.D., The Laboratory of Clinical Investigation, NIAID, Lawrence Chessin, M.D., Laboratory of Molecular Biology, NIAMD, Dr. David Davies, Arthritis and Rheumatism Branch, NIAMD, Dr. Henry Metzger.

Man Years: (computed for the 12 month period)

Total:	4 1/2
Professional:	2 1/2
Other:	2

Project Description:

Objectives: The primary objectives of this project are to study the genetics and functional characteristics of human immunoglobulins and relate these characteristics to molecular structure. Finding previously unrecognized forms or categories of immunoglobulin molecules is an important part of this work. As different forms of immunoglobulin molecules are recognized, attempts are made to detect genetic factors in order that the genetic map of structural cistrons coding for immunoglobulin polypeptide chains can be more fully developed. In addition, aspects of the chemical heterogeneity of immune globulins are being studied to improve our understanding of the mechanisms generating the heterogeneity and to determine the possible biologic significance of this heterogeneity. The different forms of immunoglobulin molecules continue to be studied for correlations between molecular structure and biologic functions such as antibody activity, reaginic activity, etc. To further understanding of the functional significance of the classes and subclasses of immunoglobulins, use will be made of these "experiments of nature" leading to disease states characterized by selective immunoglobulin deficiencies. Patients with these diseases will be studied for functional aberrations of their immune system.

Methods Employed: Studies required isolation of immunoglobulins from normal serum and also from the serum and urine of patients with multiple myeloma and macroglobulinemia. Isolation and analytic technics included ion exchange chromatography, gel filtration, preparative electrophoresis, immune precipitation in gel, autoradiography, and analytic ultracentrifugation. Protein structural studies utilized paper chromatography, high voltage electrophoresis, and the amino acid analyzer. Known human genetic factors (Gm and Inv) were detected by hemagglutination inhibition using agglutinating sera obtained from special human donors in whom these agglutinators are present. A bank of these rare agglutinating sera and special human Rh antisera has been assembled.

Major Findings: The major findings of this project report are divided into a number of separate but related areas. 1) There is a report of studies of antibody activity in myeloma proteins with cryoglobulin characteristics and an extension of these studies into the area of x-ray crystallography. Antibody activity in the subclasses of human serum IgG has also been assessed and an unusually homogeneous antibody of the IgG4 subclass is described. 2) Metabolism of immunoglobulin molecules has been studied in two patients with apparent selective hypercatabolism. In addition, a study of the metabolism of the individual subclasses of IgG has been initiated. 3) The immunoglobulin biosynthetic capabilities of human peripheral lymphocytes in short-term culture have been investigated. 4) Stimulation of lymphoblastic transformation of human lymphoid cells by antisera to human immunoglobulins has been observed. 5) The immunoglobulin carrying reaginic activity in serum from allergic patients has been characterized. 6) The effect of molecular conformation on the expression of certain immunoglobulin antigenic determinants has been studied. 7) The amino acid sequence of the amino-terminal regions of heavy polypeptide chains from IgG, IgA and IgM have been determined.

Work previously done on this project led to the description of four antigenic subclasses of IgG (IgG-1, IgG-2, IgG-3 and IgG-4). Individual myeloma proteins were found to be only of one of these four subclass types. Certain myeloma proteins have the peculiar characteristics of becoming insoluble in the cold and are known as cryoglobulins. Antigenic analysis of these cryoglobulins showed that more than one subclass of IgG was usually found in the cryoprecipitate. This raised the question of whether there was some specific interaction between the IgG cryoprotein of one subclass type and other serum IgG molecules of other subclass types. Investigation performed in collaboration with Dr. Howard Grey of the Scripps Clinic and Research Foundation have revealed evidence of specific interaction of such a nature that it appears that some cryoglobulin molecules behave as antibody toward other IgG molecules. Additional investigations are in progress to determine the number of specific antibody binding sites present on cryoglobulin molecules and, also, to determine the binding constants of such interactions.

A most important observation has been made in that some cryoglobulin preparations appeared crystalline under the microscope. Further examination of these crystals has shown birefrigence in a polarizing microscope and an attempt is being made, with the collaboration of Dr. David Davies, NIAMD, to determine whether these crystals can be made suitable for x-ray diffraction studies. As yet there have been no crystals of intact immunoglobulin molecules that have been suitable for x-ray diffraction studies; and until such crystals are produced, attempts to study the three dimensional structure of immunoglobulin molecules will be stymied.

Previous studies had shown that the IgG-1, IgG-2, and IgG-3 subclasses could contain antibody activity in normal human serums. There was no evidence showing antibody activity in the fourth subclass, IgG-4. Investigations carried out in collaboration with Dr. Burton Anderson of Northwestern University Medical School have revealed antibody directed against clotting factor VIII (AHG) in the serum of a patient with a bleeding diathesis. This IgG antibody activity has been shown to be carried by molecules of the IgG-4 subclass. This is a remarkable finding in that the antibody activity was restricted to IgG-4 molecules with k-light chains. Very few other instances of such homogeneous antibodies have been described. All of the recognized subclasses of IgG have thus now been shown to contain the functional activity of antibody.

A second major area of immunoglobulin investigation is related to the metabolism of these molecules. Two patients have been identified whose serums contained low concentrations of IgG and albumin. These patients, who are brother and sister, have been studied in collaboration with Dr. Thomas Waldmann of the Metabolism Branch, NCI. Results of studies of the catabolism of intravenously injected trace-labeled immunoglobulin and albumin preparations have revealed that both of these patients are hypercatabolizing both IgG and albumin. These are the first recorded cases of true hypercatabolism unassociated with other systemic illnesses. It is hoped that through studies of such patients insight into the mechanisms of the catabolic processes for immunoglobulin molecules may be gained.

Previous investigations from this and other laboratories have given some information about the relationship between the serum level of a given immunoglobulin and its catabolic rate. It is known, for example, that for IgG, the greater the serum concentration, the more rapid the IgG catabolic rate. The various subclasses of IgG exist in the serum at quite different concentrations, and it has not been clear whether it is the total serum IgG concentration that is important in controlling catabolic rates or whether each of the subclasses has an independent control mechanism. To answer this question a study has been initiated in collaboration with Dr. Thomas Waldmann, Metabolism Branch, NCI, to investigate the metabolism of intravenously injected representatives of the four subclasses of IgG, both in patients with normal levels of the four subclasses and in patients who have selective increases or decreases of one or more of the subclasses.

Biosynthesis of immunoglobulin molecules by human peripheral lymphocytes has also been investigated. Studies undertaken in collaboration with Dr. Lawrence Chessin, NIAID, have shown that circulating human lymphocytes from peripheral blood may synthesize all classes of immunoglobulins (IgG, IgA, IgM, IgD, type K and type L).

The parameters studied included the cell concentration necessary for detection of newly synthesized immunoglobulin molecules, the intensity, position and distribution of radiolabel in specific precipitin arcs and the number of classes and types of molecules synthesized. In addition, biosynthetic studies over time showed that cells maintained in vitro frequently retained the capacity to synthesize immunoglobulins and that they displayed biosynthetic patterns distinctive for cells from a given individual after various periods of time in culture.

Certain aspects of the lymphoblastic transformation of lymphoid cells in short-term culture have been under investigation in collaboration with Dr. Joost Oppenheim. Previous studies with Dr. Oppenheim have shown that monkey antisera to human immunoglobulins could cause the transformation of a small percentage of human lymphocytes. Antisera prepared against human immunoglobulins in other species (rabbits and goats) were incapable of stimulating human lymphocytes to transform. Further work on stimulation of human lymphocytes by monkey antisera has been discontinued since antisera which previously were effective in stimulating transformation deteriorated with storage and no longer have stimulatory activity. It has not been possible to prepare fresh antisera because of the unavailability of monkeys for immunization. One observation made in the previous study was that when the stimulating antiserum was heavily absorbed with the antigen against which it was directed, the stimulatory activity was decreased. In addition, the absorbed antiserum actually became inhibitory of the transformation of lymphocytes. Extension of these studies has shown that if rabbit antiserum to human IgG is absorbed with human IgG in antigen excess and the supernatants containing soluble complexes of rabbit anti-IgG and human IgG are tested for either stimulatory or inhibitory activity with human peripheral lymphocytes, these complex-containing supernatants are markedly inhibitory and will block the ability of human peripheral lymphocytes to transform when stimulating by mitogens such as phytohemagglutinin. These inhibitory complexes provide a tool for learning more about the biochemical processes involved in lymphocyte transformation.

Studies have been carried out in collaboration with Dr. Kimishige Ishizaka concerning the nature of reaginic antibodies and their possible relationships to the classes and subclasses of immunoglobulins. This work has clearly shown that reaginic antibody as defined by the capacity to passively sensitize human skin does not belong to any one of the previously recognized classes or subclasses of human immunoglobulin, but does represent a new immunoglobulin class. A number of investigators who have been studying reaginic antibodies, including Dr. Ishizaka and myself, met under the auspices of the World Health Organization in Lausanne, reviewed information

available concerning the structure and function of reaginic antibody and agreed that this new immunoglobulin should be designated IgE or γE.

Studies of immunoglobulin structure have been carried out in two major areas. The first relates to the importance of conformation of polypeptide chains in the formation of antigenic determinants in the human IgM molecule. It has been shown that there are certain determinants which are present in the intact molecule but which cannot be detected when the constituent polypeptide chains of the molecule are looked at separately. The polypeptide chains when recombined produce molecules that again contain these antigenic determinants in a detectable form. The development of reagent antiserums which can detect such conformation-dependent antigens, particularly where the antigenic determinant depends upon the presence of light chains of one type or another, may be of use in studying the functional characteristics of molecules of either kappa or lambda type present in the normal human serum, since there are no good methods for separating kappa from lambda molecules out of normal human serum.

The other line of structural investigation has been concerned with the primary amino acid sequence of the constituent polypeptide chains of immunoglobulin molecules. One area of investigation has been the determination of the amino terminal sequence of the heavy polypeptide chains of representative myeloma proteins from different classes and subclasses of immunoglobulins. This work has been carried out in collaboration with Dr. Leroy Hood, Immunology Branch, NCI, and Dr. Henry Metzger, Arthritis and Rheumatism Branch, NIAMD. Preliminary evidence suggests that the amino terminal sequences of the different subclasses of IgG do not differ significantly one from the other while the amino terminal sequences of IgG and IgM do seem to contain significant differences. Determination of the amino terminal sequences of IgA heavy polypeptide chains has proven technically difficult and procedures are currently being devised for isolating the appropriate peptides. This particular line of research is of theoretical importance since it has been predicted that the amino terminal regions of all of the classes of heavy chains should be essentially the same. The data being acquired in the present study should rigorously test this theoretical prediction.

Significance to Bio-Medical Research and the Program of the Institute:

The development of a more complete understanding of the operation and control of the immune system requires that the component parts of the system be identified as precisely as possible. In the past this work has depended heavily upon the utilization of myeloma proteins which can be isolated in a more homogenous state than their normal counterparts in normal human serum.

The fact that myeloma proteins appeared to lack specific antibody activity was considered to be a potential drawback in their further utilization for the structure and function of antibody molecules. The evidence that certain myeloma proteins have antibody specificity obviates this difficulty and makes the search for additional myeloma proteins with demonstrable, well-

defined antibody activity of great importance. If a number of myeloma proteins with the same apparent specificity can be identified, then it is possible that the primary structural factors responsible for generating this particular antibody specificity may be definable. In addition, if these proteins with the same specificity happen to have different binding affinities, it may be possible to determine the structural correlates for binding constants. The finding that certain myeloma-like proteins both have antibody activity and form crystals under certain circumstances, leads to the exciting possibility of obtaining crystaline antibody molecules and of being able, therefore, to determine the three-dimensional structure of antibody molecules. It is only when both three-dimensional and primary sequence information can be put together that it will be possible to fully understand the nature of the interaction between antibodies and their antigens.

Regulation of immunoglobulin synthesis can best be studied in the patient, and the investigations into metabolism of immunoglobulins both in individuals with apparently normal metabolic processes and in those with specific defects, such as hypercatabolism, should shed light on the nature of the control mechanisms exercised in the intact human organism.

Mechanisms underlying blast transformation of immunocompetent human cells such as the circulating lymphocyte are still not understood. The ability to induce transformation of these cells with antiserums directed not against the cells, but rather against a synthetic product of the cells, provides a controlled experimental system in which many aspects of lymphocyte function, DNA and RNA synthesis, and protein synthesis can be studied. The inhibition of blast transformation by immune complexes provides an additional tool for studying the various metabolic processes that are known to take place in the course of this cellular transformation. Better understanding of such cellular mechanisms are necessary before proper control and manipulation of the human immune system can be achieved.

Proof that antibody molecules of the reaginic type represent a distinct class of immunoglobulins opens the possibility of determining the structural correlates for this unique biologic activity and further establishes the possibility of altering the course of events in clinical allergic states.

The generation of molecular variability among antibody molecules is not understood. Many theoretical formulations have been proposed and some of these can be subjected to critical testing. The accumulation of information concerning the primary amino acid sequences of the variable regions of heavy chains from the different classes and subclasses of immunoglobulin molecules should establish further constraints on the types of theories that may legitimately be entertained.

Proposed Course of Project: Continued search for antigenic, functional, and genetic differences among the heavy and light polypeptide chain of human immunoglobulin molecules will be carried out.

Clinical investigations of selected patients with hypogammaglobulinemia and dysimmunoglobulinemia will be continued.

Structural correlates of antigenic, functional, and genetic characteristics will be studied by determining amino acid composition, peptide map finger print analysis, carbohydrate analysis and where indicated, amino acid sequences of appropriate immunoglobulin polypeptide chains.

The amino acid residue at position 191 of the kappa type light chains present in normal human serums will be determined. Previous work from a number of laboratories have established a relationship between the amino acid present at this position and the genetically determined serologic Inv determinant of such polypeptide chains. All of the previous work, however, has been done with isolated Bence Jones proteins or kappa-type light polypeptide chains derived from individual myeloma proteins. It is important to establish that the same relationship holds for the kappa-type light chains present in normal human serum. It is conceivable that the observations made to date represent coincidental correlations between the 191 residue and the genetic type of the protein and that the interchange at position 191 really represents two different subclasses of kappa polypeptide chains.

Deeper probes into the nature and consequence of lymphoid transformation mediated by anti-immunoglobulin antiserums will be undertaken. Studies of the specificity of the reaction, the parts of the molecules involved, and the effect of the reaction on immunoglobulin synthesis are now in progress.

Honors and Awards: None

Publications:

Ishizaka, K., Ishizaka, T., and Terry, W.D.: Antigenic structure of γE-globulin and reaginic antibody. J. Immunol. 99:849-858, Nov. 1967.

Andersen, B.R., and Terry, W.D.: γG4-globulin antibody causing inhibition of clotting factor VIII. Nature. 217:174-175, Jan. 1968.

Chessin, L.N., Glade, R.P., Asofsky, R., Baker, P.D., Reisfeld, R., and Terry, W.D.: Studies on human peripheral lymphocytes in vitro V. Biosynthesis of immunoglobulins. J. Exp. Med., in press.

Grey, H.M., Kohler, P.F., Terry, W.D., and Franklin, E.C.: Human monoclonal γG cryoglobulins with anti-γ-globulin antibody activity. J. of Clin. Inv., in press.

Kunkel, H.G., Fahey, J.L., Franklin, E.C., Osserman, E.F., and Terry, W.D.: Notation for human immunoglobulin subclasses. J. Immunol. 99:465, Aug. 1967.

Terry, W.D.: Variations in the subclasses of human IgG. Proceedings of the Third Developmental Immunology Workshop on Immunologic Deficiency Diseases in Man. in press.

Serial No. NCI-5003 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Control of Immunity

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M. D.

Other Investigators: M. Terry McEnany, M. D.

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total:	1-1/2
Professional:	1/2
Other:	1

Project Description:

Objectives: The objectives of this project are: (1) to investigate the role of humoral and cellular factors in maintaining normal immune response, (2) to define the changes occurring in disease, and (3) to manipulate these factors for therapeutic purposes. Immediate objectives have been to identify the serum immunoglobulins responsible for enhancing activity in a transplantable mouse tumor system and to improve technics for evaluating serum and cellular immune components.

Methods Employed: Patients with antibody and immunoglobulin deficiencies are selected on the basis of clinical and laboratory findings. Quantitative immunoglobulin methods, and serum antibody measurements are used. Inbred strains of mice and transplantable lymphocytic and plasmocytic tumors are used in laboratory studies. Tissue culture, mixed cell and bioassay technics are used to assess immune activity.

Major Findings: Our prior studies showed that the enhancing activity was present in the 7S immunoglobulins, and indicated that most of the activity is in the 7S γ_{2a}-globulins. The IgM, IgA and 7S γ_1-globulins are without enhancing activity. Efforts to improve the qualitative and quantitative assessment of immune enhancement and cytotoxicity have been directed at developing in vitro assays and this work has progressed slowly as several systems had to be discarded after initial trials.

Significance to Bio-Medical Research and the Program of the Institute: The enhancing serum modifies the effect histocompatibility differences. The finding that most enhancing activity is present in 7S γ_{2a}-globulins indicates that passively transferred activity will distribute widely throughout the body. (60% of γ_{2a}-globulin is extravascular) and will persist for relatively long periods. Enhancing activity of serum could be advantageously used in transplantation immunology and should be avoided in tumor immunology.

Proposed Course of Project: Efforts to establish in vitro methods using tissue culture technics, etc., will continue. Enhancement of syngeneic tumor systems will be attempted, i.e. studies of tumor rather than histocompatibility differences. Also, further efforts will be made to separate enhancing and cytotoxic antibody activities.

Preliminary investigations of human cell and serum systems will be undertaken.

Honors and Awards: None

Publications:

Irvin, G. L., III, Eustace, J. C., and Fahey, J. L.: Enhancement activity of mouse immunoglobulin classes. J. Immunol. 99: 1085-1091, Dec. 1967.

Mardiney, M. R., Jr., Montes de Oca, H., and Bennett, J. M.: Cell-Cell Interaction: The interaction of tumor and normal lymphoid cells in mixed cultures. J. Immunol. In press.

Serial No. NCI-5004 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Formation of Antibodies and Immunoglobulins

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M.D.

Other Investigators: John R. Wunderlich, M.D. and Donald N. Buell, M.D.

Cooperating Units: None

Man Years: (computed for the 12-month period)

Total:	4
Professional:	1 1/2
Others:	2 1/2

Project Description:

Objectives: Large scale in vitro synthesis of antibody for use in man is one goal of this work. Understanding the biosynthetic processes and regulatory mechanisms involved in the formation of antibodies and other immunoglobulins is another.

Methods Employed: Tissue culture of human lymphoid cell lines is an important part of the work. Specific immunochemical technics (see Project NCI-5000) and immunofluorescent cytology are used to detect immunoglobulin formation. Labeled amino acid incorporation into specific immunoglobulins is investigated in vivo, in tissue slices, in cell free systems and with purified ribosome preparations. Normal spleen, lymph nodes and bone marrow and malignant lymphoid and plasma cell tumors are obtained from patients.

Major Findings: New lymphoid cell lines synthesizing immunoglobulins were studied with special emphasis on cell lines from normal individuals or patients without malignant disease. These lines have been found to synthesize immunoglobulins of the same types as found in cell lines derived from patients with malignancy.

Additional evidence indicates that the human lymphoid cell lines, previously referred to as Burkitt tumor lines, leukemia lines, etc. may not represent Burkitt tumor, leukemia, etc. We have suggested that these may represent a common type of cell, possibly a normal cell which in culture does not represent the patients' malignancy in any way.

Detailed studies of immunoglobulin-producing cell lines indicate that some cells produce two forms of heavy chain. This feature of some in vitro cultures is very rarely encountered in normal or malignant cells in vivo.

No relation was found between presence or absence of herpes-like virus and immunoglobulin production in the human lymphoid cell lines.

Significance to Bio-Medical Research and the Program of the Institute: The original finding that human immunoglobulins (γ-globulins) can be formed in tissue culture systems has been confirmed and extended. Materials are now available to attempt to induce antibody formation by human cells in vitro.

The expression of multiple genes for immunoglobulin production in vitro (in contrast, to single genes in vivo) indicates that special conditions exist in the tissue culture situation. These should provide an opportunity to explore in a new manner the regulatory mechanisms for gene expression and antibody formation.

The evidence that a normal or malignant stem cell has been successfully cultured encourages efforts to influence culture conditions so that specific lines of cell development may be encouraged. It seems reasonable to propose that erythroid, myeloid or megakaryocytic cells could be derived from stem cells that may also develop into lymphoid cells.

Proposed Course of Project: Studies are being undertaken to determine: 1) if antibody is being produced by the tissue culture cells, 2) if the culture lines can be "instructed" to make specific antibody, 3) if the function of the cells can be influenced by environment. Regulator substances will be sought.

Honors and Awards: None

Publications:

Finegold, I., Fahey, J.L., and Granger, H.: Synthesis of immunoglobulins by human cell lines in tissue culture. J. Immunol. 99: 839-848, Nov. 1967.

Finegold, I., Fahey, J.L., and Dutcher, T.F.: Immunofluorescent studies of immunoglobulins in human lymphoid cells in continuous culture. J. Immunol. in press.

Finegold, I., Hirshaut, Y., and Fahey, J.L.: Immunochemical and morphologic comparison of donor tissues with immunoglobulin producing tissue culture lines from two patients. Cancer Research in press.

Fahey, J.L. and Finegold, I.: Synthesis of immunoglobulins in human lymphoid cell lines. Proceedings of the 32nd Cold Spring Harbor Symposium. Cold Spring Harbor, L. I., New York, June, 1967, pp. 283-289.

Serial No. NCI-5005 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Investigations of Malignant Lymphocytic and Plasmocytic Diseases

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M.D.

Other Investigators: Daniel Ein, M.D. and John R. Wunderlich, M.D.

Cooperating Units: None

Man Years: (computed for the 12-month period)

Total:	1 1/2
Professional:	1
Others:	1/2

Project Description:

Objectives: Malignant reticular and lymphomatous diseases are investigated with a view to determining the mechanisms responsible for the major disabling features of these illnesses, to clarify diagnosis and prognosis, and to improve treatment. Investigations have focused on Waldenström's macroglobulinemia, multiple myeloma, heavy-chain disease, and related disorders with abnormal serum and urine immunoglobulins.

Methods Employed: Physicochemical and immunochemical technics are employed for quantitative assessment of changes in immunoglobulins and other serum proteins. Quantitative immunologic technics are used to measure response to antigenic stimulation. Histologic evaluation is conducted by histochemical, immunohistologic, electron microscopic and standard morphologic procedures. Clinical evaluation is based on specific protocols derived from previous experience.

Major Findings: One patient with heavy-chain disease was identified and studied in some detail. He had many findings of severe rheumatoid arthritis but none of malignant lymphoma. Biosynthetic and immunofluorescent studies demonstrated that the H-chains were formed in the bone marrow and lymphoid tissue.

Two patients with non-macroglobulinemic hyperviscosity syndrome were studied. One had a G-myeloma protein that aggregated at 37°C to form macromolecules (larger than 135) which caused a high blood viscosity. The other had a very high 7 S G-myeloma protein level (12 grams %) with consequent elevation of serum viscosity. Both patients had clinical symptoms attributable to hyperviscosity which responded to reduction in serum IgG levels.

Studies with patients with D-myeloma protein were extended and the results correlated.

Significance to Bio-Medical Research and the Program of the Institute: Biosynthetic and immunofluorescent technics are being used to correlate functional features with clinical and morphologic findings in lymphocytic and plasmocytic malignancies.

Distinction between cases of multiple myeloma based on the type of myeloma protein are indicated by the initial observations in patients with D-myeloma proteins, and patients with two or more myeloma proteins. Further observations are needed, however, to extend these studies.

Investigations in patients with Waldenström's macroglobulinemia indicate that this disease, with marked increases of specific serum immunoglobulin, has a clinical course that typically differs from multiple myeloma. Search for additional therapeutic and prognostic differences within the general categories of lymphocytic and plasmocytic diseases continue.

Proposed Course of Project: Efforts to identify the significant clinical features of multiple myeloma will be extended in patients with D-myeloma proteins, E-myeloma proteins and heavy-chain disease.

Clinical observations and therapeutic evaluation of macroglobulinemic lymphoma will be continued.

The hyperviscosity syndrome is characteristic of macroglobulinemia and rarely, other hyperglobulinemia. The mechanisms through which hyperviscosity of the blood produces clinical effects is not understood. Studies on blood flow, oxygenation, etc. are being undertaken to investigate this problem.

Honors and Awards: None

Publications

Fahey, J.L., Carbone, P.P., Rowe, D.S., and Bachmann, R.: Plasma cell myeloma with D-myeloma proteins (IgD myeloma). Am. J. Med. in press.

Serial No. NCI-5006 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Transplantation Antigens

Previous Serial Number: Same

Principal Investigator: G. Nicholas Rogentine, Jr., M.D.

Other Investigators: Dean L. Mann, M.D., Timothy Canty, M.D., and John L. Fahey, M.D.

Cooperating Units: Germ-free Laboratory, NIAID, Drs. Richard Asofsky and Mark Mandel

Man Years: (computed for the 12 month period)

Total:	2 1/2
Professional:	1
Other:	1 1/2

Project Description:

Objectives: The objectives of this study are: a) to identify and quantitate antigens of normal and malignant leukocytes with particular emphasis on antigens of the HL-A (human leukocyte alloantigen) system and the H-2 alloantigen system of the mouse; b) to prepare these antigens, thought to be transplantation antigens, in soluble subcellular form; c) to chemically and immunologically analyze these soluble antigens; d) to use these subcellular transplantation antigens in biological tests of their ability to provoke homograft rejection or specific immunological tolerance with the ultimate goal of inducing tolerance in human transplantation; d) to investigate the metabolism of subcellular transplantation antigens.

Methods Employed: ^{51}Cr release and dye exclusion cytotoxicity (see Project Report NCI-5008) and a radiolabeled antibody test were used to detect alloantigens. Human cells were obtained from normal blood bank donors and from tissue culture. Tumor cells, lymph node and spleen cells were used for assaying mouse H-2 alloantigens. Antisera detecting genetically determined human leukocyte alloantigens were obtained as described in Project NCI-5008. Mouse alloantisera were prepared in the conventional way.

Human leukocyte alloantigens in a soluble form were prepared by the method of Mann et. al. (see publications). The method involves graded hypotonic lysis of cell suspensions, recovery of lipoprotein membrane fragments from the supernatant and papain digestion of these fragments to yield a water soluble antigen. Mouse H-2 antigens were prepared by the method of Nathenson, essentially the same method as Mann et. al., and were supplied to us by Dr. Stanley Nathenson, Albert Einstein College of Medicine, New York.

Major Findings: Techniques to detect and quantitate HL-A alloantigens on normal and malignant peripheral blood leukocytes and on human tissue culture cell lines using immune cytolysis have been established (see Project Report NCI-5008).

Attempts to develop a direct test for cellular antigen quantitation utilizing radioactive-labeled human alloantisera were unsuccessful due to the high amount of nonspecific binding of normal human sera to the cells.

Water soluble human HL-A antigens were obtained from two human lymphoid tissue culture cell lines known to contain a broad spectrum of different HL-A antigens (see Project Report NCI-5008). Soluble preparations were also obtained from peripheral blood leukocytes and from spleen cells. These glycoprotein preparations were applied to Sephadex G-150. Two major peaks of alloantigens were found. The minor peak had a M.W. of >200,000; the major peak had a M.W. of about 60,000. Together these two peaks contained all of the HL-A antigens tested for in the cell from which they were derived. When this human material was combined with solubilized mouse H-2 antigens both products chromatographed in the same region on Sephadex G-150, indicating physicochemical similarity between the human HL-A antigens, thought to be transplantation antigens, and the mouse H-2 antigens, known to be transplantation antigens. Further purification and biological testing of this human material is in progress.

In collaboration with Dr. Stanley Nathenson soluble mouse H-2 alloantigens were tested for their ability to provoke homograft immunity. Prior immunization with these antigens either intravenously or intraperitoneally with Freund's adjuvant resulted in accelerated graft rejection indicating that these soluble antigens are transplantation antigens.

The soluble mouse H-2 antigens in as low a dose as 10^{-2} µgm were found to induce immunological tolerance as indicated by significant suppression of the Simonsen splenomegaly assay. Thus, these antigens are capable of both provoking homograft immunity and invoking tolerance.

Significance to Bio-Medical Research and the Program of the Institute:

The preparation and analysis of soluble human transplantation antigens has both basic and clinical significance. Basic information concerning the relationship of antigenic determinants to chemical structure will help in understanding the complex genetics of the HL-A locus. Clinically, soluble

transplantation antigens may be used to induce specific tolerance to homografts and thus may obviate the need for hazardous immunosuppressive agents.

Studies of cellular interactions in normal growth and differentiation and in neoplastic transformation have formed increasingly on the cell membrane. Immunological evaluation of membrane antigens such as the important HL-A antigens can yield precise qualitative and quantitative information about cellular interaction. If antigenic abnormalities are detected in malignant cells, for example, there may form a basis for immunotherapy or chemotherapy of malignant disease.

Proposed Course of Project: Further biological and biochemical characterization of soluble HL-A antigens will be accomplished. After suitable preclinical testing of the pharmacology and toxicology of these preparations in animals, biological experimentation will be undertaken in patients. These biological experiments will be directed at answering two questions; a) are these antigens immunogenic and b) are they tolerogenic? If tolerance can be demonstrated, these antigens will be used in human hematopoietic cell transplantation (see Project Report NCI-5008) to ameliorate or prevent both graft rejection and graft-versus-host disease.

These soluble human alloantigens will be used to immunize rabbits or other animals in an attempt to produce specific human typing reagents.

Further biological studies relating to the induction and maintenance of tolerance with soluble mouse histocompatibility antigens and the metabolism of these antigens will be carried out.

Because of the limitations of commonly available methods for detecting cell antigens, continued work in developing and applying new methods is needed. In particular, the C'1s fixation and transfer technique of Borsos and Rapp and the indirect immunofluorescent technique will be adapted for use with human cells and human alloantisera. The potential of these two methods for quantitative analyses of antigen sites on cell membranes and for assessing antigens on single cells is a strong reason for their development.

Honors and Awards: None

Publications:

Mann, D.L., Rogentine, G.N., Jr., Fahey, J.L. and Nathenson, S.G.: Solubilization of Human Leucocyte Membrane Isoantigens. Nature 217:1180-1181, March, 1968.

Serial No. NCI-5007 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Immunotherapy of Neoplastic Disease

Previous Serial Number: Same

Principal Investigator: John L. Fahey, M.D.

Other Investigators: Ronald B. Herberman, M.D., M. Terry McEnany, M.D., and G. Nicholas Rogentine, Jr., M.D.

Cooperating Units: Melpar Inc. Contract #PH 43-66-3
(Dr. George Olson)

Man Years: (computed for the 12-month period)

Total:	2
Professional:	1 1/2
Others:	1/2

Project Description:

Objectives: The objectives of this project are to retard, arrest and prevent tumor growth both in model systems of experimental animals and in clinical disease of man.

Methods Employed: Transplantable tumors induced by viral (polyoma virus and Moloney virus), chemical or unknown agents are used in model systems, employing C57BL/6, BALB/C and SJL and other inbred mice. Serum and cells (and cell components) of potential value in modifying tumor growth are evaluated under a variety of dosage conditions by bioassay in model systems and in human subjects.

Major Findings: An affiliated laboratory facility has been established at Melpar, Inc. through contract #PH 43-66-3. A professional investigator (Dr. George Olson), a technician and animal caretakers, plus facilities for 10,000 mice and laboratory research have been assembled since December, 1967, in order to conduct animal studies with an emphasis on pre-clinical model systems.

Some experiments have been completed with polyoma and methylcholanthrene induced tumors in C57BL/6 mice. Viable cells in subthreshold doses did induce immunity and protection against tumor challenge. Non-viable cells were with-

out benefit under the conditions explored.

The effect of adding foreign protein to tumor cells in an effort to increase tumor immunogenicity was explored (McEnany). Either rabbit gamma globulin or hemocyanin were coupled to the cells with bisdiazobenzidine. Control tissues included normal spleen cells or lethally x-irradiated tumor cells. Experiments with plasma cell tumor LPC-1 revealed no immune effect of the protein addition. Growth of melanoma S91 was delayed in animals given protein-treated tumor and protein-treated normal spleen. Similarly, x-irradiated cells (without protein) caused delayed appearance of tumors. No specific adjuvant effect can be ascribed to protein-treated tumor cells.

Significance to Bio-Medical Research and the Program of the Institute: Model systems with known tumor-specific antigens are essential for experimental attempts to improve immune response to tumor antigens.

Establishment of facilities in which to conduct adequate studies in mice make further studies feasible. Mouse systems allow studies of tumor antigens without the hazards of alloantigen variability.

The studies with modified tumor cells reveal that tumor growth can be modified by experimental manipulation of tumor cells. The evidence clearly indicates the need for carefully controlled studies if meaningful results are to be obtained. The present studies do not support the hypothesis that conjugation of proteins to tumor cells will increase their immunogenicity. They indicate that clinical trial of this approach would not be appropriate at the present time.

Proposed Course of Project: Clinical studies are being undertaken to study the effects of antiserum or cells on tumor growth. The first step is an in vivo bioassay to determine if the serum has any cytolytic effect on tumors. Subsequent to demonstration of anti-tumor effect, protocols for evaluation of immunotherapy will be developed.

Three approaches with antiserum are being explored for initial clinical studies.

(1) Antiserum directed against the HLA-1 allo(iso)antigens has been collected and assayed. Potential recipients will be typed and antiserums directed against various specific alloantigens will be employed. Active serum will be fractionated to determine if 18 S or 7 S (or other) fractions have antitumor activity.

(2) Serum from tumor patients in remission is being collected and will similarly be assayed.

(3) Use of heterologous anti-tumor or anti-lymphocyte serum is being considered. Initial clinical studies will be developed with lymphoma, leukemia, multiple myeloma and mycosis fungoides.

Similar studies will be conducted with animal models using viral and carcinogen-induced as well as other tumors. Efforts to obtain immunogenic non-viable tumor cells or cell components will be extended.

Honors and Awards: None

Publications:

Fahey, J. L.: Prospects for Immunotherapy. Cancer Bulletin, in press.

Serial No. NCI-5008 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Transplantation of Hematopoietic Cells

Previous Serial Number: Same

Principal Investigator: G. Nicholas Rogentine, Jr., M.D.

Other Investigators: Dean L. Mann, M.D., Timothy G. Canty, M.D.
John L. Fahey, M.D.

Cooperating Units: Medicine Branch, NCI
Drs. E. Henderson and S. Perry

Man Years: (computed for the 12 month period)

Total:	4
Professional:	2
Other:	2

Project Description:

Objectives: The primary objective of this study is the successful transplantation of hematopoietic cells in man. An important first step is the establishment of reliable means of histocompatibility testing in humans.

An important auxilliary objective is the establishment of animal model systems to study hematopoietic cell transplantation.

Methods Employed: Histocompatibility testing was carried out principally by studying genetically determined alloantigens on human lymphocytes. There is growing evidence that some of these antigens (the HL-A system) are transplantation antigens. These antigens were assayed in vitro by a modification of the ^{51}Cr release lymphocyte cytotoxicity technique. Other tissue typing techniques such as dye exclusion cytotoxicity and quantitative complement fixation tests are available in the laboratory.

Human leukocyte antigens are best detected by human alloantisera and we have obtained 53 antisera recognizing many of the different genetically determined leukocyte antigens from three sources: 1) multiple transfused Clinical Center patients; 2) multigravid women (National Naval Medical Center), and

3) the Serum Bank of the Transplantation Immunology Branch, NIAID.

Hematopoietic cell transplantation was studied in two animal model systems: a) the lethally irradiated adult inbred mouse given allogeneic bone marrow, and b) the newborn inbred mouse given allogeneic spleen cells (Simonsen graft-versus-host assay).

Immunosuppressive heterologous anti-mouse lymphocyte or anti-mouse thymocyte sera were prepared in rabbits by repeated intraperitoneal injections of appropriate viable cells. Anti-human lymphocyte sera were prepared in horses by repeated intradermal injections of human lymphoid tissue culture cells in complete Freund's adjuvant.

Major Findings: Nine families (35 individuals) were histocompatibility typed for possible bone marrow or peripheral white cell transplantation. In accord with present theories about the genetics of the HL-A locus (the principal leukocyte alloantigen locus), identical matches at HL-A were found among siblings. Other investigators have demonstrated prolonged skin and kidney graft survival in siblings matched at the HL-A locus. Thus, an important prerequisite for hematopoietic cell transplantation, matching for the major histocompatibility locus (HL-A), has been achieved with the ^{51}Cr release cytotoxicity technique.

Collaborative studies of tissue typing and hematopoietic cell transplantation have been undertaken in three patients. Two bone marrow transplants were done on patients with acute leukemia and marrow failure. In both instances, one on the Medicine Branch Service, NCI, and one at the Baltimore City Hospital, Baltimore, Maryland, the patient succumbed to causes related to the basic disease before proof of graft survival could be ascertained. One patient with metastatic osteogenic sarcoma on the Surgery Branch Service, NCI, was given peripheral white cells from his parents (not matched to the patient at HL-A) to determine possible antitumor effects of these allogeneic cells.

An Outpatient Clinic for patients with marrow failure was established in the Immunology Branch, NCI.

Sixteen human lymphoid tissue culture cell lines (see Project Report NCI-5004, 1966-1967) were assayed for HL-A antigens using between 21 and 40 antisera which recognize antigens at this locus. Each cell line contains one or more of these antigens. No two cell lines are the same. Detailed analyses of four of these cell lines indicate that the antigens are qualitatively maintained over periods of continuous culture of up to 14 months. The peripheral blood lymphocytes of donors of 8 of these cell lines have been assayed for HL-A antigens and compared with the HL-A antigens of their own lymphoid tissue culture cells. In five cases, identity was noted. In three others, there appeared to be more antigens present on the tissue culture cells than on lymphocytes. In the two cases that were investigated further, this difference was found to be quantitative and not qualitative. Quantitative

absorption tests with 8 antisera in these two cases revealed that the tissue culture cells had an antigen density on the cell membrane which varied from the same as the peripheral blood lymphocyte to 35 times as much. Two of these cell lines were selected for use in preparing soluble HL-A antigens (see Project Report NCI-5006).

The techniques of marrow procurement and transplantation were applied to the protection of the lethally irradiated adult mouse successfully in several strain combinations. The use of an allogeneic marrow inoculum protected the animals from early radiation death but later resulted in a reproducible, lethal graft-versus-host (GvH) syndrome. Since GvH in humans is also frequently fatal, the establishment of this model is the first step in attempting modification of GvH.

A more sensitive and quantifiable GvH assay, the Simonsen splenomegaly assay was also successfully established. Attempts at modification of GvH in this model system will be an important adjunct to studies in the radiated adult mouse.

Anti-mouse lymphocyte and thymocyte sera have been produced in rabbits and used in mice to prolong skin graft rejection. Horse anti-human lymphocyte serum is being prepared and will be used clinically after appropriate toxicology studies in animals.

Large quantities of alloantisera were collected from 15 post-partum women and from one patient with aplastic anemia all of whom had demonstrable antileukocyte antibodies.

Significance to Bio-Medical Research and the Program of the Institute: Transplantation of hematopoietic cells in man is urgently needed as a means of correcting marrow aplasia due to chemical or physical agents. It is of particular relevance to other programs in the National Cancer Institute, as marrow aplasia is often seen in patients who have received high doses of chemotherapeutic agents or radiation for malignancy. The selection of compatible donor-recipient pairs is of prime importance in assuring graft acceptance and minimizing graft-versus-host disease.

The use of newer immunosuppresive agents such as anti-lymphocyte serum may lead to greater graft success with fewer of the undesirable side effects that attend the use of other immunosuppresive agents.

One of the major problems in successful hematopoietic cell transplantation in man is the control of the graft-versus-host syndrome. Investigations along these lines are needed to overcome this stumbling block before marrow transplantation can be widely used as a treatment for marrow aplasia.

Proposed Course of Project: Patients will be admitted to the Immunology Branch for transplantation of hematopoietic cells. Bone marrow and peripheral blood leukocytes will serve as sources of transplantable cells. Donors and

recipients will be typed for leukocyte and erythrocyte antigens and compatible pairs will be selected.

The importance of animal model systems to study the effect of various immunosuppressive agents on graft rejection and on graft-versus-host disease cannot be overemphasized. Modifications of graft rejection and GvH will be attempted using antilymphocyte serum, drugs, radiation and specific soluble transplantation antigens. The mouse model systems already available will enable us to make such observations. In addition, sub-human primate models (Rhesus monkeys) will be established and similar experiments carried out in these animals. In vitro separation of sub-populations of hematopoietic cells will be attempted and the ability of the various fractions to restore marrow function will be assessed. As these avenues of research are developed, they will be adopted for transplantation efforts in man.

Honors and Awards: None

Publications:

Rogentine, G.N., Jr., and Plocinik, B.A.: Application of the ^{51}Cr cytotoxicity technique to the analysis of human lymphocyte isoantigens. Transplantation 5:1323-1333, Sept. 1967.

Rogentine, G.N., Jr.: Detection of isoantigens on human lymphocytes and tissue culture cells by the ^{51}Cr cytotoxicity technique. Curtoni, E. (Ed.): Histocompatibility Testing. Copenhagen, Denmark, Munksgaard, 1967, pp. 371-380.

Serial No. NCI-5009 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Tumor Antigens

Previous Serial Number: None

Principal Investigator: Ronald B. Herberman, M. D.

Other Investigators: John L. Fahey, M. D., Leroy Fass, M. D.

Cooperating Units: None

Man Years: (computed for the 12 month period)

Total:	2
Professional:	1
Other:	1

Project Description:

Objectives: The objectives of this project are: 1) to detect unique antigenic features of malignant cells, 2) to detect antibodies to tumor-specific antigens, 3) to evaluate the diagnostic, prognostic and therapeutic significance of antibodies to tumor antigens, 4) to detect delayed hypersensitivity reactions to tumor antigens and 5) to define normal antigens and other features of cell membranes.

Methods Employed: Serum and cells are obtained from patients and control subjects and from cells maintained in tissue culture. Inbred strains of mice and transplantable murine tumors induced by viral, chemical or unknown agents are used in model systems. Antigen-antibody reactions are measured by complement dependent cytotoxicity reactions. Other immunologic techniques are being developed. Some antibody and antigen preparations are tested by bioassay in human subjects.

Major Findings: In prior studies, we found that most normal human sera and disease sera contain a complement-dependent cytotoxic antibody which reacts with a variety of human lymphoid cell cultures, derived from patients with Burkitt's tumor and from other individuals. Studies of this antibody have been extended by correlating the presence of antibody and antibody titer with age. Activity is present in most cord sera; at 6 months to 2 years of age, it is present in only 20% of sera; thereafter, there is an increasing incidence with age, until the adult incidence and titer are reached at 14 years of age.

The antigen detected by the cytotoxicity test is being studied in some detail. Preliminary evidence indicates that: 1) the antigen is present on the lymphoid tissue culture cells derived from a variety of disease states and from normal individuals, and is, therefore, not a disease-specific antigen; 2) the antigen is not a leukocyte isoantigen; 3) it is present on all human leukocytes and other nucleated cells tested, but in 50-100 fold lower concentrations than on the tissue culture cells; 4) it is also present on nucleated cells of several mammalian species; 5) a cross-reacting antigen is present on many gram-negative bacterial strains, in the cell wall lipopolysaccharide fraction. The results indicate that human sera contain a natural antibody which cross-reacts with antigens that are widely distributed in nature.

As a model system for developing sensitive serologic techniques for tumor antibody detection, antibodies to the mouse H-2 histocompatibility antigens and the human HLA antigens have been studied. Antibodies to H-2 antigens have been detected by an indirect ^{125}I-labeled antibody technique. A five to ten fold increase in the sensitivity of cytotoxic antibody detection has been obtained by the addition of an anti-immunoglobulin reagent.

Significance to Bio-Medical Research and Program of the Institute: Detection of tumor antigens and of antibodies to tumor-specific antigens is essential for progress in tumor immunology. Tumor-specific antibodies are yet to be conclusively demonstrated in human tumor systems. Antibody activity against tumor antigens must be carefully distinguished from antibodies against blood group antigens and other cellular antigens. The cytotoxic antibodies previously detected in our studies are not tumor-specific. They are natural antibodies which have characteristics of both heterophile and autoantibodies.

Efforts to develop more sensitive serologic techniques are important because better methods are needed to detect weak tumor antigens.

Cell-mediated reactivity to tumor-specific antigens is another important aspect of tumor immunology. Detection of delayed hypersensitivity to tumor antigens should provide an in vivo method of assaying cellular immunity.

Proposed Course of Project: Studies of the natural cytotoxic antibody will be continued. Clinical correlation of activity in serial serum samples will be made in patients with leukemia, Burkitt's tumor, infectious mononucleosis and other diseases. Studies will be performed to investigate the large increase in antigenic concentration on the lymphoid culture cells relative to peripheral leukocytes. Further studies to define the biochemical nature of the antigen will also be performed.

Development of more sensitive serologic techniques for detection of anti-cell antibodies will be pursued. The radio-isotope labeled antibody technique is a particularly promising approach. Efforts will be made to understand better and reduce the large amounts of nonspecific globulin adsorption to cells, which is the major limitation of the technique.

Clinical studies in tumor immunology will be initiated. Autologous tumor cells and membrane extracts from these cells will be tested for delayed hypersensitivity reactions.

Honors and Awards: None

Publications:

Herberman, R. B., and Fahey, J. L.: Cytotoxic antibody in Burkitt's tumor and normal human serum reactive with cultures of lymphoid cells. Proc. Soc. Exp. Biol. Med. 127: 178, 1968.

Herberman, R. B.: Cytotoxic natural antibodies in human serum. In Combined Clinical Staff Conference: The Circulating Lymphocyte - Its Role in Infectious Mononucleosis. Annals Int. Med., in press.

Serial No. NCI-5010 (c)
1. Immunology Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Antibodies: Structure, Genetics and Evolution

Previous Serial Number: None

Principal Investigator: Leroy Hood, M.D., Ph. D.

Other Investigators: William D. Terry, M.D. and Daniel Ein, M.D.

Cooperating Units: Arthritis and Rheumatism Branch, NIAMD, Henry Metzger, M.D. Structural studies of human μ immunoglobulin polypeptide chains.

Man Years: (computed for the 12-month period)

Total	2
Professional:	1
Others:	1

Project Description:

Objectives: The object of this project is to define the structure of immunoglobulin (antibody) polypeptide chains and to relate this structure to the genetic origin and evolutionary development of the immune system.

Methods Employed: Immunoglobulin molecules are examined analytically by physicochemical, immunochemical and other appropriate techniques. Serum and urine are fractionated by electrophoretic, chromatographic, and gel filtration techniques. Individual polypeptide chains are prepared by chemical reduction followed by gel filtration. The resulting polypeptide chains are broken down by enzymes into smaller fragments and these fragments are further characterized by the classic techniques of amino acid sequence analysis--the Dansyl-Edman technique, hydrazinolysis and other suitable procedures.

Major Findings: This part of the report will be divided into three sections: 1) genetics of the specificity region in light chains, 2) genetics of the specificity region in heavy chains, and 3) the evolution of immunoglobulin chains.

Recent amino acid sequence studies in this and other laboratories have demonstrated that the kappa immunoglobulin light chain is divided into two separate regions--a carboxy terminal half, the common region, which is encoded by a single gene and an amino terminal half, the specificity region, which is

coded by at least three discrete germ-line genes. Proof for the multiplicity of specificity region genes rests on the fact that the kappa specificity sequences can be divided into three discrete classes (Hood and others). Dr. Ein and I have demonstrated that a similar situation exists with regard to the specificity region of lambda chain, that is three or more discrete germ-line genes must be responsible for encoding the variable regions of lambda chains. Our studies have also demonstrated that single individuals express the subclass sequences in markedly different ratios. This has important implications on the nature of the genetic machinery responsible for synthesizing these molecules.

Very little amino acid sequence information is available on the specificity regions of heavy chain hence small peptides have been isolated from the amino terminal portion of mu, gamma and alpha polypeptide chains. The sequences from the gamma chains resemble one another very closely, yet they can be distinguished quite easily from the sequences of the mu and alpha chains. The same appears to be true of each of the other sets of sequences, that is mu sequences can be readily distinguished from an alpha sequence and the alpha sequences can be readily distinguished from gamma sequences. It was interesting to note that N-terminal sequences from each of the different γG subclasses were virtually identical.

Studies on the evolution of genes determining immunoglobulin light chain are complicated by the extreme heterogeneity of the molecules and by the existence of two separate and independent gene systems, specificity region genes and common region genes. Studies of the specificity region of more than 15 different animals have demonstrated; 1) that all of these animals have lambda and/or kappa specificity regions and 2) that these animals have one or more of the subclasses normally found in humans. Limited amino acid sequence analyses have been carried out on the common regions of three primates, monkey, baboon and human, and on two artiodactyls, cow and sheep. These studies have demonstrated that the common regions of the three primates are very closely related to one another and in fact, that each of the primates has both kappa and lambda genes. In contrast, the artiodactyls have only the lambda genes. The artiodactyl genes are much more distantly related to those of the primate as one might expect from a glance at the vertebrate evolutionary tree. Analysis of this data has opened up a number of new avenues of investigation. For example, it appears that Oz-like markers (found in another approach to human immunoglobulin (Ein)), are present in the two artiodactyls, whereas they are not present in monkey and baboon.

This difference in species distribution has important genetic implications (with regard to the nature of common region genes).

Significance to Bio-Medical Research and the Program of the Institute:
The observation that the specificity region of human lambda light chains must be encoded for by three or more discrete germ-like genes places very important constraints on any theory of antibody diversity. These observations, as they stand, could be accounted for by either of the major theories of

antibody formation, the germ-line theory which states that there are many germ-line antibody genes or the somatic theory which states that there is a single (or few) germ-line antibody gene. The subclasses in both kappa and lambda chains may be used as tools to investigate these theories.

The distinct amino acid sequences found in the specificity regions of mu, alpha and gamma heavy chains suggest that separate genes must encode the specificity regions of each of these immunoglobulin polypeptide chains. Further, it strongly suggests that there is no sharing of specificity regions among immunoglobulin polypeptide chains. This places further constraints on theories of antibody diversity.

Apparently immunoglobulin molecules evolve in a fashion similar to that of other well-studied systems, e.g. cytochromes and hemoglobins. It is hoped that a study of the evolution of antibody molecules will yield some clues as to the genetic mechanism for generating antibody diversity. To date, no such information has been obtained. However, a number of interesting questions have been raised with regard to the control mechanisms which regulate antibody synthesis. For example, why is it that some animals have lambda, some animals have kappa and some animals can have both lambda and kappa light chains? Have the genes disappeared from those animals which synthesize a single class of immunoglobulin light chains, or in fact, have they been repressed in some fashion? Techniques have been developed which would permit, if necessary, the sequencing of common regions of light chains from normal individuals in various species. Such amino acid sequence information can tell us how fast the common regions of immunoglobulin light chains have evolved. Finally, a number of useful techniques have been developing for dealing with heterogeneous proteins and for making evolutionary comparisons between such proteins from various types of animals.

The combination of studies discussed above will give new insights into the nature of the genetic machinery which produces antibody diversity, into the control mechanism which regulate synthesis of antibody molecules, and into the evolution of the immunoglobulin system.

Proposed Course of Project: The genetic and structural problems noted above will be pursued in several directions. 1) Lambda light chains with unusual amino acid sequences in the specificity region will be studied in an attempt to define further subclasses of lambda specificity regions. 2) The search for a genetic marker in the specificity region of normal individuals will continue using the chemical characteristics of the different light chain subclasses as a possible marker. 3) Additional amino acid sequence data will be obtained from the specificity region of various types of heavy chains. 4) The study of the evolution of immunoglobulin molecules will be continued concentrating primarily on the common regions from light chain molecules.

Honors and Awards: None

Publications: None

ANNUAL REPORT OF THE METABOLISM BRANCH
SUMMARY OF SIGNIFICANT ACTIVITIES
NATIONAL CANCER INSTITUTE

July 1, 1967 through June 30, 1968

The Metabolism Branch consists of five senior investigators studying the physiology and biochemistry of man as affected by neoplastic diseases. Each investigator is pursuing a separate line of investigation. The patients selected for study do not necessarily have neoplastic disease, but a biochemical or physiological abnormality that is to be studied. In general, the clinical disease states selected for study have been those in which it is most likely that the physiological process can be adequately understood. In a similar way, parallel studies are carried out in experimental animals.

During the last year, an Immunophysiology Section was created, and Dr. Thomas A. Waldmann was selected to lead this section.

Amino Acid Transport

Studies of amino acid transport in bone have been extended to include tibia bone chips from six-week-old rats. It has been shown that rat tibial bone chips (TBC) differ from fetal rat calvaria (FRC) in mechanisms of amino acid transport. In FRC, neutral amino acids (alpha-aminoisobutyric acid, glycine) non-competitively inhibits the uptake of imino acids (proline, hydroxyproline). However, competitive inhibition was demonstrated between imino and neutral amino acids in TBC. This suggests that a transport carrier present in FRC is absent in TBC. Additional work is being done to elucidate the mechanism of this difference.

A multicompartmental analysis of proline-C^{14} kinetics in FRC demonstrated a non-linear dependence of collagen and protein synthesis on intracellular concentrations of proline. Hydroxylation of peptide-linked proline in collagen was the mechanism responsible for a time lag in appearance of labeled hydroxyproline. Increasing hydroxyproline concentration in the media was shown to increase the rate constant describing collagen incorporation without a change in the hydroxylation rate constant. This suggests that increased hydroxyproline concentrations stimulate collagen synthesis through an intrinsic feedback mechanism.

Calcium Metabolism

Computer multicompartmental analysis was used to study regulatory mechanisms in calcium metabolism. A saturable mechanism has been shown for gastrointestinal absorption of calcium. This was corroborated by the finding that the frequency of dietary calcium intake affected urinary excretion of calcium. Subjects showed greater urinary calciums on six divided doses of milk when compared to the same total calcium intake in a single dose.

Kinetic studies in patients with fluorosis suggested that bone was hyporesponsive to parathyroid hormone. This was further tested in fluorotic cows by monitoring plasma calcinosis and parathyroid hormone concentrations while giving constant EDTA infusions. Whereas normal cows showed a homeostatic response in plasma calcium concentrations after two hours of EDTA infusions, fluorotic animals exhibited continuing fall in plasma calcium in spite of elevated parathyroid hormone levels.

Dietary calcium perturbation of normal subjects produced a regulatory response primarily at bone resorption. However, in patients with abnormal calcification states; i.e., fluorosis and myositis ossificans, bone formation was the main regulatory mechanism. This suggests that metabolic bone disease has altered the normal intrinsic and hormonal regulatory mechanisms.

Porphyrin Metabolism

Studies of the factors controlling porphyrin biosynthesis have been continued. The mathematical model of enzyme synthesis has been studied by Dr. Waxman using computers. The use of inhibitors of protein synthesis (puromycin and actinomycin D) by intravenous administration to rats has led to calculations of the half-life of the messenger RNA for hepatic ALA synthetase. These refinements of previous cruder calculations indicate the messenger RNA to have a half-life of about 10 to 15 minutes. This verifies our previous observations that this is a messenger RNA with a very rapid turnover. Further studies on the repression of hepatic ALA synthetase by heme demonstrate that the production of the enzyme cannot be repressed completely by heme. This raises the question of two ALA synthetases, but attempts to demonstrate this by other methods have been unsuccessful. Other data on the kinetics of this system have been obtained, but not yet analyzed.

A number of drugs and synthetic compounds have been studied for their ability to induce hepatic ALA synthetase in live intact rats. Quantitative comparisons of this induction effect have been made. The previous data published by other investigators using tissue culture methods have been found to differ considerably from the findings in live animals. Although the system developed in our laboratory for screening compounds which may prove dangerous in porphyria is not perfect, it will prove to be of some value.

The mechanism of the "glucose effect," i.e., the ability of glucose to block the induction of hepatic ALA synthetase is under study. A number of metabolic intermediates derived from glucose have been studied in terms of their ability to block ALA synthetase induction. Preliminary studies indicate that while certain tricarboxylic acid cycle intermediates have some activity, none thus far studied can be administered in sufficient amount to equal the effects of glucose quantitatively. One possible exception may prove to be α-keto-glutarate. This compound, when administered to live animals, has inhibited porphyrin precursor synthesis. Possible chelation effects and the relationship of iron to the early stages of porphyrin synthesis are also now being pursued.

It has been found that the diabetic glucose tolerance curve seen in all patients during attacks of acute porphyria does not result from lack of

insulin secretion, but is actually accompanied by an insulin secretion curve similar to that of adult onset diabetes; i.e., a delayed but sometimes excessive secretion of insulin often resulting in hypoglycemia four hours after a glucose test.

The use of lithium in the treatment of depression was studied in one patient with acute intermittent porphyria. This treatment was highly successful in this one patient who had been followed for a number of years and who had been treated by other methods previously without success. The use of insulin along with high carbohydrate intake, which was previously initiated in this laboratory as a possible treatment of acute intermittent porphyria, has now been pursued in other institutions and appears to be of some value. Some patients with acute intermittent porphyria have been found to have an abnormal increase of beta lipoproteins in addition to the well documented hypercholesteremia. This is being studied further.

The Physiology and Pathophysiology of the Immune Response

Studies were directed toward determining the pathways of the circulating and cellular immune response to antigenic challenge, and to determine the nature of the immunological defects in patients with hereditary disorders associated with frequent infections and a high incidence of reticuloendothelial malignancies. The immunological defects of 12 patients with the Wiskott-Aldrich syndrome were studied. The Wiskott-Aldrich syndrome is a disorder characterized by thrombocytopenia, eczema, frequent infections, and a 25 percent incidence of reticuloendothelial neoplasms. These patients had profoundly diminished delayed hypersensitivity responses, impaired homograft rejection, but had normal circulating lymphocyte counts and normal in vitro lymphocyte transformation to phytohemagglutinin. Although they had near normal serum immunoglobulin levels, they had absent or reduced natural antibody titers to blood group antigens and five serotypes of E. coli and had diminished antibody responses to polysaccharide and protein antigens. Patients with the Wiskott-Aldrich syndrome thus have a broad immunological defect involving both humoral and cellular immune responses. Such a broad defect associated with the presence of functional lymphocytes and adequate immunoglobulin levels suggest that this is a disorder of antigen processing or recognition; that is, a disorder of the afferent limb of immunity. Ataxia telangiectasia is another syndrome with frequent infections and a high incidence of reticuloendothelial neoplasms. Patients with this disorder were also shown to have decreased circulating antibody responses to antigenic challenge and impaired ability to manifest cellular immune responses. Only one of 11 subjects with this syndrome had IgA in either the serum or the external secretions. The patients studied had a marked defect in IgA synthesis. In 2 of the patients, there was, in addition, an exceedingly short survival of IgA secondary to the development of antibodies to this molecule.

A new familial disorder involving the hypercatabolism of serum proteins was discovered. Two siblings, the product of a consanguinous marriage, had marked reduction in the serum concentrations of albumin and IgG. The total body pools of these proteins were markedly decreased, while the rate of synthesis of these proteins was normal. The fractional catabolic rate for these proteins was 3

to 5 times greater in these patients than in controls. Both patients had normal fecal ^{51}Chromium-albumin tests, excluding excessive gastrointestinal protein loss, and neither had proteinuria or hyperthyroidism; thus, these patients had a previously unrecognized familial abnormality characterized by reduction of the serum levels of IgG and albumin due to a disorder of the catabolic pathways of these patients. Hypogammaglobulinemia secondary to disorders of immunoglobulin catabolism was also seen in 25 patients with myotonic dystrophy who had an isolated hypercatabolism of IgG.

Eleven of the 85 patients with thymomas were shown to have a marked reduction of serum concentration of IgG. Seven of these patients had a reduction of all classes of immunoglobulin molecules. These patients had an inability to respond with circulating antibodies to antigenic challenge and had delayed hypersensitivity defects. All of these patients with thymoma and hypogammaglobulinemia had the association of the absence of circulating and marrow eosinophils. No eosinophils could be demonstrated in the local inflammatory response studied by the Rebuck skin window technique even when fibrinogen was used as a stimulus for eosinophil migration. When such studies were performed in control subjects, eosinophils were the predominant cell in the exudate. The patients had normal urinary 17-keto and hydroxy steroid excretions, and thus did not have increased adrenal function as the cause of their eosinopenia. Other patients with agammaglobulinemia, absence of the thymus, tumors of the thymus, or disorders of delayed hypersensitivity responses, did not have eosinopenia, and thus the eosinopenia appears to be uniquely associated with the syndrome of hypogammaglobulinemia and thymoma.

Studies of patients with gastrointestinal protein loss were continued. Three hundred studies with ^{51}Chromium-albumin have been performed. There was a close correlation between the ^{51}Chromium-albumin clearance and the fraction of the plasma pool catabolized per day over normal as estimated with iodinated albumin, a finding adding validity to the ^{51}Chromium-albumin test. Excessive gastrointestinal protein loss was demonstrated for the first time in patients with vascular disease of the bowel, tricuspid regurgitation, Sjögren's syndrome, and factitious diarrhea secondary to excessive ingestion of cathartics. Patients with intestinal lymphangiectasia, constrictive pericarditis, Whipple's disease, and those with chylous effusions were shown to have marked lymphocytopenia. Prior to therapy, these patients were capable of making normal circulating antibody responses, but had a marked defect in delayed hypersensitivity responses and could not reject homologous skin grafts over the five-year period of observation. When the primary disease was cured by pericardectomy, antibiotic treatment of the patients with Whipple's disease or the use of a very low fat diet in patients with intestinal lymphangiectasia, the lymphocyte levels and the ability to manifest delayed hypersensitivity returned to normal. Since these patients have marked anergy, but only a slight increased incidence of infections, they are a model of considerable importance in the study of organ homotransplantation.

Patients with cystinosis and disorders of the proximal renal tubule were shown to excrete high concentrations of small molecular weight proteins, including L-chains in the urine. The kidney was shown to play a major role in the catabolism of L-chains from immunoglobulins. It is felt that the

patients with cystinosis are losing small molecular weight proteins into the urine because they are being filtered normally through the glomerulus, but they are not being transported and catabolized by the diseased proximal convoluted tubule.

Erythropoiesis

Studies of the factors influencing rates of red blood cell production and destruction have been directed toward the further refinement of computer-assisted techniques of multicompartmental analysis and their application of the analysis of iron and bilirubin turnover data. A multicompartmental model of body iron distribution and turnover is being tested with data obtained from normal volunteers and patients with a variety of erythropoietic disorders. The model provides quantitative data about plasma and red cell iron turnover, red cell renewal rates and hence, indirectly, about red blood cell life span. These data agree with those which are obtained by older and more laborious methods. The model has the dual advantage of being simpler and more rapid--and hence more applicable to clinical use--than older methods, as well as providing additional information not otherwise obtainable. The sideroachrestic anemias have been selected for detailed study since they represent a situation in which erythropoiesis is markedly disturbed, and in which older methods suggest a large amount of ineffective erythropoiesis. Quantitative studies, utilizing an eight-compartment model, confirm the presence of a significant amount of ineffective erythropoiesis with subsequent recycling of iron from RBC precursors to the plasma in patients with this disorder.

Bilirubin Metabolism

Since the bulk of bilirubin production results from senescent red blood cells, bilirubin turnover studies were initially pursued as a means of quantitating erythropoiesis. They have been further developed as a diagnostic tool in the evaluation of liver disease. Bilirubin clearance studies in 40 patients have indicated that the clearance curve of bilirubin-^{14}C consists of three to four exponential components, and a multicompartmental model compatible with this finding has been developed. The model permits calculation of the sizes of the plasma, hepatic, and extravascular pools of unconjugated bilirubin, intercompartmental transfer rates, the rate of bilirubin production, and the red cell life span. It has been found that characteristic changes in model parameters occur in Gilbert's syndrome and possibly in other conditions. The bilirubin-^{14}C clearance test, therefore, may be extremely useful in the differential diagnosis of unconjugated hyperbilirubinemia of obscure etiology. Estimates of the RBC life span, based on plasma bilirubin clearance data, have been found to agree with those derived from formal RBC life span studies. Extensive clinical studies of bilirubin metabolism in normal volunteers and in patients with Gilbert's syndrome are currently in progress.

Serial No. NCI-4002(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of the Physiology and Pathophysiology of the Immune Response and of Plasma Protein Metabolism

Previous Serial Number: SAME

Principal Investigator: Thomas A. Waldmann, M.D.

Other Investigators: Warren Strober, M.D., Michael Blaese, M.D., Dale McFarlin, M.D., John Clough, M.D., and William Terry, M.D.

Cooperating Units: Metabolic Neurology Branch, NINDB, and Immunology Branch, NCI

Man Years

Total: 6
Professional: 6
Other: 0

Project Description:

Objectives: Studies were directed toward determining the pathways of the circulating and cellular immune response to antigenic challenge, to determine the nature of the immunological defects in patients with agammaglobulinemia, intestinal lymphangiectasia, ataxia telangiectasia, Wiskott-Aldrich syndrome, thymoma and hypogammaglobulinemia, and familial idiopathic hypercatabolism of serum proteins; and to determine the pathophysiology of the high incidence of reticuloendothelial malignancies seen with these disorders. Other objectives of the project were to determine the factors controlling synthesis, transport and catabolism of the plasma proteins, especially the immunoglobulins in normal subjects, patients with neoplastic disease, and patients with hereditary disorders of immunoglobulin metabolism.

Methods Employed: The rates of synthesis, catabolism and distribution of the serum proteins were studied, using radio-labeled purified proteins. Albumin and the five major subgroups of immunoglobulins, IgG, IgA, IgM, IgD, and Bence-Jones proteins, were isolated from the serum, urine, or salivary secretions, using physical-chemical procedures, including DEAE

cellulose, Sephadex chromatography, zone electrophoresis, and preparative acrylamide gel electrophoresis. The isolated purified proteins were labeled with ^{131}I, ^{125}I, and ^{51}Cr, and injected intravenously into patients and experimental animals. The curves of radioactivity in the serum, urine, stool, and nasal and salivary secretions were used with multicompartmental and mathematical models to determine the rates of protein synthesis, catabolism, and distribution into the extravascular compartments and external secretions.

The C'1a fixation and transfer test of Borsos and Rapp was modified to determine the number of molecules of IgG, IgA, and IgM antibodies synthesized by man and experimental animals in response to antigenic challenge. In studies directed toward determining the mechanism of the immune response, ribonucleic acid from macrophages cultured in vitro and exposed to antigens was incubated with lymphocytes to produce a primary antibody response in vitro.

Major Findings: Studies on the immunological defects in patients with Wiskott-Aldrich syndrome and ataxia telangiectasia were continued. The Wiskott-Aldrich syndrome is characterized by thrombocytopenia, eczema, frequent infections, and 25 percent incidence of reticuloendothelial neoplasms. In the 12 patients studied, delayed hypersensitivity responses were absent when tested with five skin test antigens and dinitrochlorobenzene sensitization, despite the fact that the patients had a normal circulating lymphocyte count and normal in vitro lymphocyte transformation to phytohemagglutinin. IgG levels were normal, IgA and IgD levels were elevated, and IgM levels were diminished due to reduced IgM synthesis. Despite these normal or near-normal levels of immunoglobulins, natural antibodies to blood group antigens and five serotypes of Escherichia coli were strikingly diminished. In addition, antibody response to polysaccharide antigens were almost completely absent. Antibody responses to a variety of bacterial, protein, and viral antigens were also significantly impaired.

We conclude that the Wiskott-Aldrich patients have a broad immunological defect involving both humoral and cellular immune responses. Such a broad defect associated with the presence of functional lymphocytes and adequate immunoglobulin levels suggest that this is a disorder of antigen processing or recognition that is a disorder of the afferent limb of immunity. These subjects have progressive hyperplasia of the reticulum cells and reduction of the lymphoid elements of the pericortical areas of the lymph nodes. Three of the 12 patients developed reticuloendothelial neoplasms during the past year. It is felt that this high incidence of reticuloendothelial neoplasms in these patients was secondary to two factors: 1) The chronic overstimulation of reticuloendothelial system by antigen that cannot be processed in the normal fashion; and 2) the inability to manifest delayed hypersensitivity responses and to reject neoplastic tissues that have minor histocompatibility differences from the host.

Studies on the immunological defect of patients with ataxia telangiectasia were continued. The patients had decreased antibody response to antigenic challenge and decreased ability to manifest cellular immune responses. Ten of 11 subjects studied with this syndrome had absence of the immunoglobulin IgA in both serum and the salivary and nasal secretions. The absence of this IgA in the external secretions appears to be an adequate explanation for the high incidence of sinopulmonary infections noted with these patients, since IgA is normally the major antibody molecule of these secretions. The patients were shown to have a marked defect in IgA synthesis, as estimated with iodinated IgA turnover studies. In two of the subjects, there was also an exceedingly short survival of IgA secondary to the development of antibodies to this molecule. The demonstration of development of antibodies to a normal serum protein may make it impossible to replace the deficient proteins in such subjects with dysgammaglobulinemia by parenteral administration of these molecules, and more significantly, may explain the frequent severe reactions to blood products and gamma globulin seen in patients with ataxia telangiectasia and other patients deficient in one class of immunoglobulin.

A new familial disorder involving hypercatabolism of IgG and albumin was discovered in studies performed in conjunction with Dr. William Terry. Two siblings, the product of a consanguinous marriage, were studied because of marked reduction of IgG, 1.3 and 4.4 milligrams per milliliter compared to 12 milligrams in control, associated with hypoalbuminemia. The metabolism of iodinated IgG and albumin was studied in these two patients and 15 controls. The total body pools of albumin and IgG were markedly decreased, while the rate of synthesis of these proteins was normal. The fractional catabolic rate for these proteins was increased three- to fivefold. Both patients had normal fecal ^{51}Cr-albumin tests excluding excessive gastrointestinal protein loss, and neither had proteinuria or hyperthyroidism. Thus, these patients have a previously unrecognized familial disorder characterized by a reduction of the levels of serum IgG and albumin due to a disorder of the catabolic pathways of these proteins. Hypogammaglobulinemia secondary to disorders of immunoglobulin catabolism were also seen in 25 patients with myotonic dystrophy who had an isolated hypercatabolism of IgG.

Eleven of 85 patients with thymoma were shown to have a marked reduction in the serum concentration of IgG. Two of these patients had normal IgA and IgM concentrations. Two of the remaining patients with lymphoid thymic tumors had reduced IgA concentration, but markedly elevated IgM concentrations. The remaining seven patients had a reduction of all classes of immunoglobulin molecules associated with spindle cell thymic tumors. These abnormalities of immunoglobulin concentration were due to decreased immunoglobulin synthesis. In addition, secondary gastrointestinal protein loss was present in one subject. All of the patients with thymoma and hypogammaglobulinemia had the unique association of an absence of circulating and marrow eosinophils. No eosinophils could be demonstrated in the Rebuck skin window studies of localized inflammation, even when fibrinogen was used as the stimulus to eosinophil migration.

When such studies were performed in control subjects, the eosinophils were the predominant cell in the exudate. The patients did not have increased 17-keto- or hydroxy-steroid excretion, and thus did not have increased adrenal function as a reason for their eosinopenia. Other patients with agammaglobulinemia, absence of the thymus, tumors of the thymus, or disorders of delayed or circulating hypersensitivity did not have eosinopenia, and thus, the eosinopenia appears to be uniquely associated with the syndrome of thymoma and hypogammaglobulinemia. Hypogammaglobulinemia due to reduced immunoglobulin synthesis was also demonstrated in patients with Bruton's type sex-linked agammaglobulinemia, idiopathic acquired hypogammaglobulinemia and in patients with amyloidosis excreting Bence Jones protein in the urine.

Studies on patients with gastrointestinal protein loss were continued. Three hundred studies with ^{51}Cr-albumin have been performed. There was a close correlation between the ^{51}Cr-albumin clearance and the fraction of the plasma pool catabolized per day over normal as estimated with iodinated albumin, thus adding validity to the ^{51}Cr-albumin test. Excessive gastrointestinal protein loss was demonstrated for the first time in patients with vascular disease of the bowel, tricuspid regurgitation, Sjogren's syndrome, and factitious diarrhea secondary to excessive ingestion of cathartics. The studies of immunological status of patients with gastrointestinal protein loss secondary to disorders of the lymphatic channels of the bowel were continued. Patients with intestinal lymphangiectasia, constrictive pericarditis, and Whipple's disease were shown to have both hypogammaglobulinemia and marked lymphocytopenia secondary to loss of lymph into the gastrointestinal lumen. Prior to therapy, these subjects were capable of making normal circulating antibody responses, but had a marked defect in delayed hypersensitivity responses and could not reject homologous skin grafts over the five-year period of observation. When the primary disease was cured by pericardectomy, treatment of the patients who had Whipple's disease with antibiotics or the use of a very low fat diet in patients with intestinal lymphangiectasia, lymphocyte levels and the ability to manifest normal delayed hypersensitivity returned to normal. A similar lymphocytopenia and inability to reject skin homografts has now been demonstrated in patients with lymphocytopenia secondary to chylous effusions without loss of proteins or lymphocytes into the gastrointestinal tract.

In previous studies, we have shown that the kidney plays a major role in the catabolism of Bence Jones proteins and the L-polypeptide chains of the immunoglobulins. The urine of patients with cystinosis was studied and shown to contain a great number of small molecular weight proteins demonstrable by acrylamide gel electrophoresis. The patients with cystinosis were found to excrete 20 times the normal level of lambda and kappa types L-chain in their urine. It is felt that normal subjects filter a large number of small molecular weight proteins through the glomerulus and that they are transported into and catabolized in the proximal convoluted tubule of the kidney. When there is a defect in tubular function, these proteins appear in the urine.

Significance to Bio-medical Research and the Program of the Institute: A number of animal tumors have been shown to have specific tumor antigens. The study of patients with immunological deficiency syndromes and a high incidence of neoplastic disease may be of value in understanding the pathways of the immune response and the errors of immunity that lead to the development of neoplastic disease. The common feature in the syndrome associated with a high incidence of neoplasm is a disorder of delayed hypersensitivity. The present studies indicate that reduced immunoglobulin levels may occur, not only to reduced immunoglobulin production, as in subjects with thymoma and hypogammaglobulinemia, but that excessive loss into the gastrointestinal tract and hypercatabolism may be major factors in reduced immunoglobulin levels. The anergy seen in subjects with intestinal lymphangiectasia, Whipple's disease, constrictive pericarditis, tricuspid regurgitation, and chylous effusions not associated with gastrointestinal protein loss may provide a model of value in the study of homograft transplantation.

Proposed Course: Continue studies directed toward understanding the pathways and controlling mechanisms of the synthesis, transport and catabolism of the serum proteins. Experimental models with protein and lymphocyte loss into the gastrointestinal tract will be studied with an ultimate goal of organ transplantation. Studies in vitro of the macrophage-lymphocyte interactions involved in antigen processing and antibody production will be continued and applied to human tissues in order to study the normal pathways of the immune response and the pathophysiology of immunological deficiency diseases. The sequence of IgG, IgM, and IgA antibody response in the serum and external secretions will be performed using the C'1a fixation and transfer test in order to understand better the mechanism of the circulating antibody response.

Honors and Awards

None

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Waldmann, T. A., and Strober, W.: Kinetic studies of immunoglobulin metabolism in immunologic deficiency. In Good, Miescher and Smith (Eds.): Proceedings of the Third Developmental Immunology Workshop on the Immunologic Deficiency Diseases, New York, New York, February 1-5, 1967. The National Foundation. (In press).

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Mott, P. D., Wochner, R. D., and Wolff, S. M.: The effect of 6-mercaptopurine on the turnover of I^{131} gamma globulin and I^{125} albumin in rabbits. J. Pharmacol. Exp. Ther. (In press).

Serial No. NCI-4003(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Porphyrin Metabolism in the Tumor Bearing Host and Porphyria

Previous Serial Number: SAME

Principal Investigator: Donald P. Tschudy, M.D.

Other Investigators: Annie Collins, Jeffrey A. Stein, M.D., Donald Schalch, M.D., and Kenneth Greenspan, M.D.

Cooperating Units: Laboratory of Clinical Science, NIMH; School of Medicine, Rochester University, Rochester, New York

Man Years

Total: 3 3/4
Professional: 2
Others: 1 3/4

Project Description:

Objectives: To study the mechanisms controlling porphyrin biosynthesis, particularly mechanisms of induction of the rate controlling enzyme. To investigate mathematical models of enzyme synthesis and degradation. To study biochemical and clinical aspects of the porphyrias. To apply the knowledge gained from the above objectives to the study of tumors and tumor host relationships.

Methods Employed: (A) Quantitative enzyme determinations. (B) Chemical determination of porphyrins and porphyrin precursors. (C) Use of tissue homogenates, slices and isolated mitochondria in studying metabolic pathways.

Major Findings: The mathematical model of enzyme synthesis has been studied by Dr. Waxman using computers. The use of inhibitors of protein synthesis (puromycin and actinomycin D) by intravenous administration to rats has led to calculations of the half-life of the messenger RNA for hepatic ALA synthetase. These refinements of previous cruder calculations indicate the messenger RNA to have a half-life of about 10 to 15 minutes. This verifies our previous observations that this is a messenger RNA with a very rapid turnover. Further studies on the

repression of hepatic ALA synthetase by heme demonstrate that the production of the enzyme can not be repressed completely by heme. This raises the question of two ALA synthetases, but attempts to demonstrate this by other methods have been unsuccessful. Other data on the kinetics of this system have been obtained but not yet analyzed.

A number of drugs and synthetic compounds have been studied for their ability to induce hepatic ALA synthetase in live intact rats. Quantitative comparisons of this induction effect have been made. The previous data published by other investigators using tissue culture methods have been found to differ considerably from the findings in live animals. Although the system developed in our laboratory for screening compounds which may prove dangerous in porphyria is not perfect, it will prove to be of some value.

The mechanism of the "glucose effect," i.e., the ability of glucose to block the induction of hepatic ALA synthetase, is under study. A number of metabolic intermediates derived from glucose have been studied in terms of their ability to block ALA synthetase induction. Preliminary studies indicate that while certain tricarboxylic acid cycle intermediates have some activity, none thus far studied can be administered in sufficient amount to equal the effects of glucose quantitatively. One possible exception may prove to be α-keto-glutarate. This compound when administered to live animals has inhibited porphyrin precursor synthesis. Possible chelation effects and the relationship of iron to the early stages of porphyrin synthesis are also now being pursued.

It has been found that the diabetic glucose tolerance curve seen in all patients during attacks of acute porphyria does not result from lack of insulin secretion, but is actually accompanied by an insulin secretion curve similar to that of adult onset diabetes, i.e., a delayed but sometimes excessive secretion of insulin often resulting in hypoglycemia four hours after a glucose test.

The use of lithium in the treatment of depression was studied in one patient with acute intermittent porphyria. This treatment was highly successful in this one patient who had been followed for a number of years and who had been treated by other methods previously without success. The use of insulin along with high carbohydrate intake which was previously initiated in this laboratory as a possible treatment of acute intermittent porphyria has now been pursued in other institutions and appears to be of some value.

Some patients with acute intermittent porphyria have been found to have an abnormal increase of beta lipoproteins in addition to the well-documented hypercholesteremia. This is being studied further.

Significance to Bio-medical Research and the Program of the Institute: Mechanisms of the control of metabolic pathways and the synthesis of enzymes in normal and diseased tissues are being elucidated. These control mechanisms will be investigated in tumors.

Proposed Course: (A) To continue studies of the factors controlling heme and heme-enzyme synthesis in normal and pathologic tissues. (B) To continue studies of biochemical abnormalities of acute porphyria in man and experimental animals. (C) To study control mechanisms of heme synthesis in tumors and the livers of tumor-bearing hosts.

Honors and Awards

None

Publications:

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Serial No. NCI-4004(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Amino Acid Transport and Protein Synthesis

Previous Serial Number: SAME

Principal Investigator: James M. Phang, M.D.

Other Investigator: Theodore J. Hahn, M.D.

Cooperating Units: None

Man Years

Total: 3
Professional: 2
Other: 1

Project Description:

Objectives: The aims of this project are to elucidate the membrane phenomena for active transport and to understand regulatory mechanism for cellular uptake of amino acids and their incorporation into protein.

Methods Employed: Radioisotopically labeled amino acids have been used to study in vitro uptake by rat fetal calvaria and by tibial diaphysical bone chips isolated from six-week-old rats. Alpha-aminoisobutyric acid-C^{14} has been used to study transport, and proline-C^{14} has been useful in studying the effects of purified parathyroid hormone, Vitamin D, and growth hormone on transport of amino acids and collagen synthesis.

Multicompartmental analysis of the kinetics of amino acid uptake and protein synthesis has been done on a Univac 1108 system using the SAAM 24 program.

Major Findings: Previous work from this project has shown separate transport mechanisms for neutral and imino acids in fetal rat calvaria (FRC). However, tibial bone chips (TBC) transport amino acids in a manner distinctly different from FRC. It was found that competitive inhibition could be demonstrated in TBC for transport of proline and glycine, whereas proline inhibits noncompetitively with glycine in FRC. It was also found in FRC that parathyroid hormone inhibited collagen incorporation of proline, but was without effect on

intracellular proline pools. In addition, increasing media calcium concentrations reproduced this effect suggesting that PTH is acting through a change in calcium concentration.

Multicompartmental analysis of the kinetics of proline uptake and protein synthesis showed a dependence of proline incorporation into protein upon intracellular proline pools. However, this dependence was nonlinear suggesting that the intracellular pool was further compartmented. A definite time delay in hydroxylation of peptide-linked proline was demonstrated. This delay was consistent with an enzymatic step. Furthermore, hydroxyproline stimulated incorporation of proline into collagen suggesting a feedback mechanism between breakdown and synthesis of collagen.

Significance to Bio-medical Research and the Program of the Institute: The basic biochemical mechanisms and hormone regulation of protein synthesis and amino transport must be defined to understand these processes in tumors. Collagen synthesis in primary and metastatic osseous tumors and tissue culture of malignant cell lines can be studied to define regulatory mechanisms for protein production and neoplastic growth.

Proposed Course: The difference in transport mechanisms between FRC and TBC must be further characterized. The effect of hormones, age, and predominant cell types on mechanisms of transport will be studied. Additional studies with multicompartmental analysis of uptake and protein synthesis kinetics can point out regulatory mechanisms on a molecular level.

Honors and Awards

None

Publications:

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Serial No. NCI-4009(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: A Study of the Factors Controlling Red Blood Cell Synthesis and Destruction

Previous Serial Number: SAME

Principal Investigator: Nathaniel I. Berlin, M.D.

Other Investigators: Paul D. Berk, M.D., and Robert B. Howe, M.D.

Cooperating Units: None

Man Years

Total: 4
Professional: 4
Others: 0

Project Description:

Objectives: To study the factors influencing rates of red blood cell production and destruction in normal and pathological states in man, the dog and the rat, with particular emphasis on the role of ineffective erythropoiesis in the overall body economy of heme. Studies of hepatic physiology are also conducted as an outgrowth of the use of bilirubin-^{14}C to quantitate erythropoiesis.

Methods Employed: Erythropoiesis is quantitated by the following techniques: whole blood and red cell volumes are determined with ^{51}Cr-labeled red blood cells; red cell life span is determined with either ^{14}C glycine incorporation or tritiated diisopropylfluorophosphate labeling; plasma and red blood cell iron turnovers are calculated from the plasma clearance of ^{59}Fe-labeled transferrin, and subsequent incorporation of radio-iron into circulating erythrocytes; ^{14}C-labeled bilirubin is prepared biosynthetically from glycine or delta-aminolevulinic acid; bilirubin production rate, as well as intercompartmental rate constants in the bilirubin excretory pathway, is determined by analysis of the plasma ^{14}C bilirubin disappearance curve. Ineffective erythropoiesis is estimated from the incorporation of ^{14}C label into fecal urobilinogen during the first ten days after IV administration of ^{14}C glycine.

Major Findings: The principal findings of the current year result from the expansion and further development of the computer assisted techniques of multicompartmental analysis introduced previously, and their application to the analysis of iron and bilirubin turnover data.

A multicompartmental model of body iron distribution and turnover has been developed from kinetic studies of the metabolism of ^{59}Iron in patients and normal volunteers. The model provides quantitative data about plasma and red cell iron turnover, red cell renewal rates, and hence, indirectly, about red blood cell life span. These data agree with those which are obtained by older and more laborious methods. The model has the dual advantage of being simpler and more rapid - and hence more applicable to clinical use - than older methods, as well as providing additional information not otherwise obtainable. The sideroachrestic anemias have been selected for detailed study, since they represent a situation in which erythropoiesis is markedly disturbed, and in which older methods suggest a large amount of ineffective erythropoiesis. Quantitative studies, utilizing an eight-compartment model, confirm the presence of a significant amount of ineffective erythropoiesis with subsequent recycling of iron from RBC precursors to the plasma in patients with this disorder. In the past year, analysis of additional studies, in normal subjects and a variety of disease states, has increased our understanding of iron metabolism and of the properties of the model under development.

Further studies of the metabolism of bilirubin-^{14}C have indicated that the plasma clearance curve of this substance is more complex than previously realized. In addition to the two exponential components described earlier, theoretical considerations led to a search for two additional components. One of these has been well characterized during the past year, and evidence is being gathered which supports the proposed existence of the second. These components are thought to arise from the exchange of albumin-bound bilirubin between the plasma and the two extravascular albumin compartments. Extravascular/intravascular exchange rates and extravascular pool sizes based on the analysis of bilirubin clearance data have been found to agree with similar parameters determined with isotopically labeled albumin. The presence of these additional exponential components has required the addition of suitable compartments to the multicompartmental model of bilirubin metabolism.

Significance to Bio-medical Research and the Program of the Institute: These studies are directed toward: (1) Improving the understanding of abnormalities of RBC production associated with a variety of systemic illnesses, including neoplasia; and (2) the development of clinically useful diagnostic studies through improvement and modification of the iron and bilirubin clearance studies.

Proposed Course: It is proposed to continue the studies of ineffective erythropoiesis and hepatic physiology in the experimental animal and man. These studies can be divided into three areas: 1) Continuation of the development of methods for measuring bile pigment production; 2) studies of alterations of hepatic bilirubin metabolism in various disease states; and 3) concomitantly, an investigation of ineffective erythropoiesis in patients with sideroachrestic and other selected forms of anemia.

Honors and Awards

None

Publications:

Barrett, P. V. D., Berk, P. D., Menken, M., and Berlin, N. I.: Bilirubin turnover studies in normal and pathological states utilizing bilirubin-C^{14}. Ann. Intern. Med. 68: 355-377, Feb. 1968.

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Serial No. NCI-4013(c)
1. Office of the Clinical Director
2. Metabolism Branch
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Studies of Calcium Metabolism in Malignant Disease and Metabolic Disease of Bone

Previous Serial Number: SAME

Principal Investigator: James M. Phang, M.D.

Other Investigators: Arthur N. Kales, M.D. and Mones Berman, Ph.D.

Cooperating Units: Office of Mathematical Research, NIAMD

Man Years

Total: 6
Professional: 3
Others: 3

Project Description:

Objectives: This project is designed to investigate calcium metabolism in normal man as well as in patients with neoplastic and metabolic bone disease. Physiologic and pathophysiologic regulatory mechanisms have been emphasized.

Methods Employed: Serum Ca^{47} disappearance and excretion into urine and feces are combined with metabolic balance data in a computer multicompartmental analysis to give steady state rate constants and numerical values for calcium metabolic parameters. The physiologic hypotheses suggested by this analysis are tested in animal experiments and in additional studies of calcium metabolism in man. Where applicable, parathyroid hormone levels by radioimmunoassay, bone histology, and bone X-ray crystallography are obtained.

Major Findings: Previous work on this project suggested saturable mechanisms for gastrointestinal absorption of calcium. This was tested by varying the frequency of calcium intake in the diet. It was found that normal subjects on six feedings of milk increased their urinary calcium over a single feeding of the same amount of milk with the rest of the diet kept constant. Thus, balance and kinetic work in osteoporosis and other metabolic bone diseases treated with high calcium diet must have the frequency of intake controlled.

Studies of patients with fluorosis suggested that fluoride produced a resistance to parathyroid hormone in bone. Three cows were made fluorotic to test this hypothesis. With constant EDTA infusions, these fluorotic cows showed no homeostatic response in plasma calcium in spite of rising parathyroid hormone levels, whereas matched control cows showed a rise in serum calcium by the second hour. This finding supports the hypothesis suggested by multicompartmental analysis.

Studies of calcium dietary deprivation in normal subjects and in patients with metabolic bone disease showed that normal individuals respond to low calcium intakes by increasing resorption of bone. In patients with fluorosis and myositis ossificans, bone accretion is the main regulatory mechanism. This suggests that the intrinsic and hormonal regulatory responses have been perturbed by the disease process.

Significance to Bio-medical Research and the Program of the Institute: The frequency of skeletal involvement with metastatic tumors is well known. The occurrence of hypercalcemia and osteoporosis in neoplastic diseases demands a better understanding of parathyroid hormone-target organ relationships.

Proposed Course: A regulatory model has been formulated, and studies in disease states have been used to define the fine structure of hormone secretion and target organ response. It is hoped that the effects of malignant disease on calcium metabolism may be predicted by this model.

Honors and Awards

None

Publications:

Neer, R. M., Berman, M., Fisher, L., and Rosenberg, L. E.: Calcium kinetics in man. I. Multicompartmental analysis in normal adult males. J. Clin. Invest. 46: 1364-1379, Aug. 1967.

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ANNUAL REPORT SUMMARY

SURGERY BRANCH

NATIONAL CANCER INSTITUTE

April 1, 1967 - March 31, 1968

The Surgery Branch of the National Cancer Institute continues to act in a dual capacity carrying out its own specific clinical and laboratory investigations as well as providing consultative surgical service to both the other branches of the Cancer Institute and the clinical units of the other Institutes. This surgical consultative service includes urologic, otolaryngologic, gynecologic, thoracic and general surgical consultations and when coupled with the clinical activity of the Surgery Branch requires approximately 50% of the working time of the six general surgeons, the urologist and the gynecologist of the Surgery Branch senior staff.

During the previous twelve months, 1132 written surgical consultation requests were received and answered by the Surgery Branch staff; 343 of these were from the National Cancer Institute medical units while 789 were from clinical units of the other Institutes. Each request entails on an average, five visits to the patients. Two hundred and ninty-five operative procedures were performed as a result of these consultations; 145 on patients from the Cancer Institute, 140 on patients from other Institutes. Consultants from the community saw 308 patients on our request. Two hundred and ninety-four of these patients were seen for otolaryngological problems during 85 individual visits by a team of consultant otolaryngologists.

In the past year our staff performed 725 surgical procedures; 402 major and 323 minor. Of this total, 295 were performed as a consultative service and 440 as a result of the clinical investigative program of the Surgery Branch itself. An analysis of these clinical endeavors is detailed in the tables accompanying the first section of the Annual Report protocols.

Clinical Cancer Studies

The percentage of treatment failures following conventional modes of cancer therapy have shown little change over the past five years. It is these treatment failure patients who have been referred to the Surgery Branch for study and treatment. They constitute 73% of the admissions to the Surgery Branch of the Cancer Institute. While a majority of these patients have had previous radiation and/or surgery, we are concerned by the increasing number of patients referred who have been treated primarily with other than conventional means of therapy. The use of chemotherapeutic agents, cryosurgery, chemosurgery and the laser as a primary means of therapy is apparently being introduced prematurely into the therapeutic armamentarium of some of the physicians treating malignant disease entities. The Surgery Branch has demonstrated that an appreciable number of patients with persistent malignant

disease can be offered an opportunity of cure if they have anatomically localized disease. With improved techniques of preoperative evaluation and physician and nursing care and better understanding of physiologic stress, control of infection, intrinsic chemical alteration and volume replacement, the mortality and morbidity from surgery has been significantly reduced. Frequently these patients can be cured of their cancer and returned to their family and community as responsible, wage-earning individuals.

Head and Neck Cancer

Of primary interest to the surgical clinical program, has been the patient with malignant disease of the head and neck. Ninety-one percent of all patients seen with cancer of the oral, laryngeal or pharyngeal area have a history of prolonged alcholism. These patients are also frequently users of tobacco in one form or another. Radical resection of the primary tumor-bearing area incontinuity with a simultaneously performed unilateral or bilateral radical neck dissection results in a definitive cure for many of these patients. The en bloc resection of paranasal sinus disease through a combined cranial-facial approach is now a standardized procedure in selected patients. The role of preoperative radiation in untreated malignant disease is presently being investigated with favorable preliminary results. Post-operative morbidity has been significantly reduced and in the past 53 months no operative death has occurred in surgery of the head and neck area.

Through the excellent cooperation of the dentists in the National Institute of Dental Research, cosmetic restoration of these patients has been possible. Increased interest has been directed to the cosmetic restoration of certain defects through the use of the patient's own tissue. While plastic restoration is not usually performed until that time when recurrent disease seems unlikely, there is an increasing trend towards earlier reconstruction.

Pelvic Organ Cancer

The second most commonly occurring malignant disease in females is carcinoma of the uterine cervix. The failure of these patients to have had a Papanicolaou smear continues to be a distressing aspect of this disease. Of interest has been the results in the past 27 patients in whom we were planning radical pelvic surgery, but who had scalene node biopsy taken preoperatively. Without any other evidence of disseminated disease, 3 of these 27 patients have been found to have metastatic deposits of squamous cell carcinoma in the left scalene-node bearing area. The ileal conduit continues to be the most satisfactory means of urinary diversion following total pelvic exenteration. This technique generally results in few complications and preserves excellent renal function. However, attention must be directed toward continued antibacterial prophylaxis in order to prevent infection.

Our experience with 120 radical hysterectomies, 52 anterior exenterations, 250 total pelvic exenterations and 5 combined amputative procedures (3 of which are alive for more than 4 years) suggest that this procedure should be offered to more patients with advanced pelvic disease. With intensive colostomy and urinary ileostomy instruction and guidance and vaginal

reconstruction in selected instances, these patients who have had radical pelvic surgery can be restored to essentially a normal, pleasant and productive life. However, total pelvic exenteration is a time-consuming procedure and it is doubtful that it will soon find a place in even the majority of the larger medical centers.

Extremity Sarcoma

Thirty-one hemipelvectomies and five forequarter amputations have been now successfully performed without operative mortality. Nearly all of these patients have had previous attempts at local excision of their soft-tissue sarcoma or radiotherapeutic approach of their bone tumor. If local excision is indicated, the origin and insertion of all muscle and fascial bundles encompassed by the tumor or by the previous surgical approach must be excised. With proper planning of incisions, wound healing is a minimal problem and the excellent prosthetic appliances now available, enable these patients to return to work. Patients requiring less radical amputative procedures are seldom referred, but experience indicates that bone tumors must be amputated at a level inclusive of the joint above the lesion and that soft tissue tumors must be radically excised locally if amputative procedure is not indicated or accepted.

Melanoma

An increasing number of patients with malignant melanoma are being referred and accepted for study. They all have had excision of the primary tumor and are referred because of local recurrence, inadequate margins, or regional tumor dissemination. The practice of wide, local excision with grafting and incontinuity resection of the lymph node bearing areas has been established throughout the country and is being studied in this group of patients. Many of these patients as well as several of our rectal and colon cancer patients, have demonstrated through immunological techniques an anti-tumor antibody which seems to be specific for the tumor. These preliminary findings are not yet reported and are part of an intensive and increasingly interesting part of our clinical program.

Continuing progress is being made in the elucidation of the physiologic and metabolic changes associated with extensive cancer surgery. Previously the marked contraction of plasma volume and total circulating protein together with significant decrease in extracellular fluid volume have been documented in association with extensive surgical procedures. These changes, when untreated, are associated with all the clinical signs and symptoms of hypovolemia and are corrected by the administration intra- and postoperatively of larger amounts of plasma and balanced salt solutions than the patient's measured blood loss would indicate. The clinical benefits of this therapy are quite apparent, but working out the actual mechanisms responsible continues to be an important effort in the Surgery Branch. By means of isotope dilution techniques, blood volume, plasma volume, red cell mass, functional extracellular fluid volume and total exchangeable albumin pool have been measured in patients, both preoperatively and postoperatively. In addition return of

albumin and fluid via the thoracic duct lymph has been measured. From these data it is clear that there is a reduction in the available total exchangeable albumin pool which is compatible in extent with the observed decreases in plasma volume and functional extracellular fluid volume. Experiments using isotope labelling techniques which measure the amount of protein loss into the GI tract lumen and into the gut wall are underway, and it seems very likely that the sequestered ECF and albumin will be found to reside in the wall of the intestine as a result of operative manipulation. Some of this also may be loss into the lumen of the bowel, again as a result of the change in bowel function due to manipulation. Once the quantitative documentation of this has progressed satisfactorily, it may then be possible to devise methods for correcting or alleviating this gastro-intestinal sequestration. The accomplishment of this goal will not only improve supportive therapy for maintenance of normovolemia postoperatively, but reduction of the amount of edema in the GI tract should hasten return of normal peristaltic function and lead to prompt functional recovery of patients following extensive surgical procedures.

Since the transition of neoplastic disease from a localized surgically removable lesion to systemic disease by metastases is a major factor limiting the treatment of cancer by surgery, the study of various aspects of the metastatic process continues. In several tumor-host systems in inbred mice, standardization of models with respect to both growth of primary tumor and pulmonary metastatic spread has been accomplished. Since these are spontaneous metastases rather than take and growth of a single injection of intravenous tumor cell suspension, and also represent syngeneic tumors in inbred mice, these models will be much more useful than many of the systems previously studied. In two of these systems replicate experiments have very clearly shown the efficacy of anticoagulant therapy with coumadin maintained at safe levels of anticoagulation, in producing a significant reduction in both the number of animals in whom metastases occurred and in the amount of metastatic disease occurring in those animals who do develop metastases. Additional information of possible significance is the observation that the primary tumors are smaller in the anticoagulated animals and the histopathologic picture in these primary tumors is different. This particular effect of anticoagulation is presently actively being explored since as demonstrably beneficial effects on primary tumors produced by something as non-toxic as coumadin anticoagulation may well be readily adapted to the clinical situation.

The immunological approach to problems of metastasis has been productive during the past year. Studies using the transplantation resistance method for assessing immunogenicity of tumor have been carried out to test the hypothesis that there is an immunogenic difference between primary tumors and their metastases. The results show consistent differences in immunogenicity between primary tumors and pulmonary metastases resulting from them. However, the nature of the difference depends upon the particular tumor system used and varies from tumor to tumor. These results have potential significance for the future of an immunotherapeutic approach of cancer, since the immune phenomena of the metastatic deposits as well as those of the primary tumor will have to be considered.

Investigations concerning immunological approaches to treatment of cancer continue to be an important aspect of effort in the Surgery Branch. Since these reactions posess a selectivity for disseminated small deposits of tumor cells that is not presently available by either operative surgery, irradiation, or present chemotherapeutic agents, the potential therapeutic gain from the solution of this problem is great. The search for tumor-host systems in inbred animals more closely comparable to human systems than those presently available continues. A basic problem with the standard carcinogen-induced mouse fibrosarcoma is that of trying to determine the normal non-malignant cell counterpart of this tumor. In addition, size considerations restrict experimental manipulations in mice. Two satisfactory tumors have been obtained, however, and one of them studied in some detail thus far. One, a bile duct adenocarcinoma of the inbred guinea pig, has been found to be immunogenic when tested by several different methods. In addition, immunity may be demonstrated by delayed cutaneous hypersensitivity, a simpler and quicker method of ascertaining immunity than the transplantation resistance method. So far not studied, but with considerable promise for the future, is the character of the metastases from this tumor. It spreads first to regional lymph nodes and then to the lungs, as do many human adenocarcinomas in contra distinction to many mouse tumors which do not involve regional lymph nodes with metastatic tumor. The other new tumor currently under investigation is an osteogenic sarcoma transplantable in an inbred rat strain. This tumor also metastasizes, grows well subcutaneously, and is currently undergoing evaluation of its immunogenicity. As the evaluation of these tumors progresses, the search for other model systems continues actively.

Data mentioned in this report last year, suggesting the likelihood that complement is uninvolved in syngeneic tumor immunity, have been substantiated by further experiments in a different and more clear-cut experimental design. Several tumors have been tested in congenic-resistant complement - deficient and complement containing mice, and in a F_1 hybrid situation where hybrids with a single common parent (thus permitting them to accept tumor initiated in that parent) either have normal complement or are complement deficient. Syngeneic tumor immunity is equally demonstrable in normal and in complement deficient mice. This is of considerable interest since it more strongly implicates cellular immunity as the mechanism for syngeneic tumor immunity and tends to decrease the importance of circulating humoral antibody. In addition the data suggest that the previously accepted syngeneity of the two congenic-resistant lines of mice is actually not the case. The experimental demonstration that very weak degrees of histoincompatibility may be demonstrated more clearly by sensitizing with repeated tumor growth is expected to prove useful in tissue transplantation studies where knowledge of the exact degree of histoincompatibility present is often crucial in the experiment.

Review of histologic material has been completed in a study designed to test the hypothesis that retention in situ of apparently tumor-free regional lymph nodes is important in preserving host immunologic reactivity against the tumor. The results clearly show that retention of these regional lymph nodes is of no consequence one way or another insofar as subsequent growth of residual tumor left behind at operation is concerned. The basic criteria for adequate

surgical removal of cancer continues to be just that - complete surgical removal of cancer. Since tumor in most human cases metastasizes to the regional nodes, it follows from this data that the classic concept of removal of primary tumor and regional nodes en bloc is still the desired goal.

Progress has been made toward understanding of some of the immunologic factors involved in one of the most useful experimental models for human tumor, the virus induced mouse mammary carcinoma. In this system it has been clearly shown in the past that, while the tumor is immunogenic when studied under appropriate conditions, growth of this tumor nevertheless proceeds because of immunologic tolerance in the host as a result of exposure to the inciting virus prior to this host's development of immunologic maturity. The efforts along these lines during the past year have been directed toward a clarification of the experimental situation, since there are at least six different mouse strains in which vertical transmission of mammary tumor virus occurs. The problem of further immunologic investigation of the nature of the tolerance mentioned previously would be greatly simplified were it possible to demonstrate that these viruses share common antigenicity in a fashion similar to polyoma virus strains and murine leukemia viruses. Efforts to demonstrate this with tumor transplantation resistance methods have failed so far. Little or no immunity could be shown in mice subsequent to temporary growth and surgical excision of viable tumor. This raises the problem of the relevance of persisting viremia in maintenance of tolerance to immunogenic tumor. This is a matter of some importance in considering immunotherapy for human cancer of resumed virus etiology, since persistence of the virus could easily negate attempts directed against the tumor alone. Progress has been made in studying the common antigenicity of mammary tumor viruses using in vitro techniques. Antibody against the mammary tumor virus (or antibody to mammary tumor virus induced tumor) has been demonstrated in vitro using the anti-globulin consumption method. In these studies, the anti-sera have been raised by immunizing virus free animals with allogeneic tumor due to the mammary tumor virus. The tumor graft is rejected by the usual homograft mechanisms but the virus persists sufficiently to produce antibodies. In the studies completed thus far, anti-sera induced with the C3H virus have been found to react against C3H mammary tumor virus induced mouse mammary tumors, whether these tumors developed in C3H or two other strains of mice. In addition, lactating breast homogenates from mice infected with virus have been shown to react with the antibody; whereas lactating mammary tissue homogenates from virus free animals do not. This development opens two exciting possibilities. The first is the use of this method as a prompt and reliable assay for the presence of mammary tumor virus, a tool long lacking in mouse mammary tumor investigations. The second is that the results demonstrated with this method strongly suggest that the various mouse mammary tumor viruses will indeed show immunologic cross-reactivity and may be dealt with as a single entity in this regard.

Further information supporting cross-reactivity of mouse mammary tumor viruses has come from experiments in which neonatal infection with mammary tumor virus from one strain appears to induce immunologic tolerance to tumors induced by the virus from another strain and vice versa. These data also strengthen the concept that immunologic tolerance is of critical importance in the persistent growth of virus induced neoplasms. Attempts to break this tolerance by

immunologic means, using as an immunizing substance mammary tumor cells coupled to a heterologous antigen, have been unsuccessful. These data are in accord with similar experiments using different tumor systems carried out in another branch of the institute. It would seem that this method of immunotherapy against cancer, although intriguing and promising in theory, does not work out in practice.

The second aspect of the effort directed toward further immunologic understanding of neoplastic disease has been the studies with human neoplasms. Antibody to human cancer has been found by two different methods. First has been the use of immunofluorescent techniques in which tumor cells from the patient are reacted with his sera, serum from other patients with similar neoplasms, and serum from normal individuals. The cells are then washed and fluorescent antibody directed nonspecifically against human immunoglobulins is added. By this indirect technique, fluorescence indicates the presence of antibody against the cell membrane. With this method, anti-melanoma antibody has been demonstrated in more than 60% of tests using serum from patients with melanoma and in only 20% of tests using normal sera. The antibody nature and specificity of these reactions has been verified. Particularly interesting is the significant amount of cross reactivity between antibody from one patient and the other tumors tested, and conversely between one tumor and sera from several different melanoma patients. This suggests the presence of an antigen common to all these tumors and, by analogy from animal systems, suggests that melanoma may be virus induced, since all animal neoplasms induced by the same oncogenic virus contain similar cross reacting antigens.

The other method of demonstrating antibody developed in the cancer bearing patient against his own tumor has been the lymphocyte transformation test. In this method, peripheral blood lymphocytes from patients are cultured in media containing the patient's own serum, serum from other patients, or serum from other species. Tumor cell suspension or acellular extracts are prepared and added to the lymphocyte culture. At varying periods thereafter tritiated thymidine is added to the culture, the uptake of isotope quantitatively measured, and its specific localization demonstrated with radio-autography. The lymphocytes from these tumor patients show a significant amount of transformation as judged from both uptake of tritiated thymidine and by radio-autography. This reaction does not occur against normal muscle cells from the cancer bearing patient. It is of some interest that special methods for isolation of the lymphocytes for culture have had to be developed, since many of the substances commonly used for this purpose may non-specifically stimulate lymphocytes to transform. Both of these investigations will be continued and the long-term results should be of even greater interest, since by both methods repeated assessments can be made of the anti-tumor status of the patient following surgery of other varieties of treatment. In addition these methods offer possibility of repeated assessment of the efficacy of any immunotherapeutic approach that might be developed.

Studies of the feasibility of intensive chemotherapy with methotrexate utilizing regional protection with citrovorum factor have been concluded. Microscopic examination of bone marrow specimens from the area being "protected" by regional infusion of citrovorum factor while methotrexate was

given by continuous intravenous infusion did not clearly show a protective effect on the marrow itself. In the monkeys so studied there was significant evidence for functional marrow protection. This was manifested by smaller decreases in peripheral lymphocyte and leukocyte counts and much prompter return to normal following cessation of methotrexate therapy. This was also clearly shown by decreased fatality due to the same doses of methotrexate when given to animals with regional marrow infusions of citrovorum factor as compared to animals receiving no citrovorum factor. Additionally the benefit from use of a non-absorbable intestinal antibiotic for amelioration of the gastro-intestinal toxicity of methotrexate has proven quite striking. The toxicity of internal carotid artery infusions of methotrexate was studied with the similar use of regional bone marrow infusion of citrovorum factor. Again specific effects on the regional marrow could not be demonstrated by histologic study of the marrow, but toxicity and lethality were significantly different in the regional marrow protected animals. Similar findings in this study were noted concerning the beneficial effects of gastro-intestinal antibiotics. Final review of the CNS tissue from the monkeys did not demonstrate a lesion attributable to methotrexate as compared to the control animals. While not immediately clinically applicable, these studies suggest that when more effective chemotherapeutic agents are available, combinations of either diffuse systemic administration of drug with regional protection of bone marrow or regional protection of marrow plus local intra-arterial administration of drug may be therapeutic manipulations of promise.

Studies in the Surgery Branch on the effects of laser energy on neoplastic and normal tissue have continued. The results during the past year have more clearly identified the mechanisms of action of high energy laser impulses on tumor tissue, and have increased our understanding of the adverse effects of laser as a potential modality for treatment of neoplastic disease. Much material has been subjected to histopathologic scrutiny and a clear description of the mechanism of the lasered lesion can now be made. At the epicenter of the impact there is complete vaporization and absence of tissue with cavitation in this area. As the beam passes into the tissue surrounding the epicenter the temperature is not sufficiently high to cause vaporization but superheating of the tissue fluid causes vacuolization and expansion. Due to the more rapid dissipation and escape of gas, the nearby cells are cooled with resulting partial or complete preservation and viability of some of the cell structure. In the peripheral zone of impact effect, the temperature produced is less, with neither vaporization nor superheating, but this temperature is sufficiently high and lasts long enough to produce intense cellular destruction with coagulation necrosis rather than vaporization. There are 3 concentric areas around the point of maximum concentration of the laser energy -- the first is an area of complete destruction of tumor by vaporization, the second area is one of partial distortion but incomplete tumor kill, and the third is an area of complete kill due to coagulation necrosis. The problem with further clinical application of the laser that this poses is obvious. Although apparent gross kill of tumor is assumed, when the animals are followed a sufficient period of time regrowth of tumor occurs from viable cells remaining in Zone II. With the highest amount of neodymium laser energy available from the devices used during this past year, complete destruction of even relatively small subcapsular liver nodules in rabbits has not been possible because of the

phenomena mentioned above.

Preliminary data are available on the effect of laser on adjacent tissue outside Zone III From studies in normal and partially hepatectomized rats. While analyses of these data are not complete as yet, it is apparent that there is no real difference in the capacity of the cells outside Zone III to bring about regeneration of cells in the impact site.

Some of the physical changes occurring in the center of tumor being treated with laser energy have been measured. Simultaneous measurements of both temperature and pressure have been carried out. With a thousand joule pulse from the neodymium laser, the steel sheath and the thermocouple placed 6 mm. deep to the surface focus of the laser beam were melted. Since the melting point of stainless steel is about 1500° C, it is assumed that even a higher temperature is generated by this laser pulse. The pressures associated with this have been found to range from 300-800 lbs. per sq. in. The adverse effects of this high temperature have been described above. The adverse effect of the impact pressure is the displacement of viable cells into adjacent normal tissue.

In addition to these studies of the acute effects of laser energy on tumor and adjacent tissues, follow-up of animals previously lasered continues for evaluation of long-term side effects of treatment with the laser. To date some 200 mice have been followed for over two years after treatment of dorsal flank tumor with laser. Fifteen of these mice developed neurologic bladder dysfunction which lead to their death, but no other abnormalities were noted. A group of monkeys lasered 2½ years ago remain healthy and well.

The cooperative effort between engineer and physician continues in attempts to modify the physical characteristics of the laser beam in such a way as to minimize adverse effects and develop devices which will allow delivery of cancericidal laser energy in a fashion more useful for clinical cancer therapy.

Even though obstruction at all levels of the urinary tract is a matter of prime concern to the urologist, obstructions proximal to the ureteropelvic junction have thus far received little attention. Recently we have recognized a new syndrome characterized by nephralgia due to intrarenal vascular obstruction of the superior infundibulum. In patients with this syndrome, urinary tract infection due to stasis may be associated with pain. The anatomical details in this syndrome are difficult to elucidate using standard methods of urologic investigation, but new methods combining angiographic and pyelographic techniques with radioactive scanning techniques and cine radiography, have greatly facilitated the preoperative study and diagnosis of these patients. The techniques developed for surgical correction of these abnormalities will be useful in dealing with other intrarenal abnormalities such as stones, small tumors and segmental pyelonephritis. Fruitful collaboration with the Diagnostic Radiology Department has also been evident in studies of patients with anorchidism. By selective renal arteriography the intra-abdominal testes may be precisely located and the presence or absence of tumor demonstrated. Continuous slow intra-arterial infusion of epinephrine into the renal artery by percutaneous angiographic catheter techniques has been shown to have a

singularly protective effect on the kidney receiving the infusion during radiation therapy to both kidneys. Clinical applications of this method for radiation protection of the kidney are many, and we will pursue with special interest the utility of this method in studies of renal homotransplantation. Prototype units of artificial, implantable external urinary sphincters have been developed and are currently undergoing evaluation in dogs. As these studies are completed the use of this device in patients with incontinence is expected to follow shortly.

The techniques of the molecular biologist have been applied in the investigation of small animal models of human genito-urinary disease. Changes in the subcellular environment of renal cells following acute ureteral obstruction, varying degrees of renal ischemia and contralateral nephrectomy have been outlined. In the obstructed mouse kidney, there is an abnormality in RNA metabolism consisting of a progressive disorganization of the polyribosomes and an inhibition of ribosomal RNA synthesis. Ischemia also causes a change in the classes of polyribosomes consisting of a decrease in heavy, rapidly sedimenting polysomes and an increase in single ribosomes. These changes are apparently caused, at least in part, by the activation of an intracellular nucleolytic system by ischemia. Nephrectomy causes a prompt (within 2 hours) reorganization of the polyribosomes of the remaining kidney. The sudden biochemical changes found in the contralateral kidney suggest that the stimulus to compensatory hypertrophy is present soon after nephrectomy. Eventually, it is hoped that the subcellular environment of irreversibly damaged renal cells can be characterized. This information may then be applicable to determining irreversible damage to stored kidneys or kidneys to be transplanted.

Similar studies of polyribosome biochemistry are being performed using a new tumor model. This tumor model was developed by transforming hamster prostatic tissue with SV40, a DNA oncogenic virus. The transformed cells, when transplanted, produce tumors that grow rapidly and kill the host within 2-3 weeks. The tumors metastasize readily and produce elevated levels of serum tartrate inhibitable acid phosphatase (TIAP). Tumor growth is suppressed by estrogen. Also, the serum TIAP levels in tumor bearing animals are returned to normal by estrogen treatment. The polyribosomal profile from this tumor is unique in that the polyribosomes exist mainly as single and oligo-ribosomes with very few heavy, rapidly sedimenting ribosomes. Unlike most other animal or plant cells the percentage of total cellular RNA existing as membrane bound ribosomes is only about 5%. The amount of nonsedimenting RNA is higher in these tumor cells than normal cells. Isolation of individual ribosomes from this tumor and studies of their response to EDTA, which binds all available magnesium, has shown that these tumor ribosomes completely disassociate into smaller subunits with much less vigorous chemical treatment than is usually required to produce similar disassociation of normal ribosomes. These investigations are felt to be especially meaningful since in few other instances are there animal models for human neoplasms which so closely resemble the human situation not only morphologically and biologically, but also biochemically.

In cooperation with the Pathologic Anatomy Branch certain aspects of viral oncogenesis are continuing as significant areas of investigations. One such fruitful result, as mentioned above, is the establishment of a trans-

plantable prostatic tumor resembling in many important respects its human counterpart. Efforts are underway to produce a series of primate tumors growing in the autochthonous host by removing the pineal gland from adult Virvet monkeys, establishing an explant culture in vitro, transforming of this tissue with SV40 virus and returning the transformed pineal cells to the original host. The first aspect of this difficult but very rewarding study has been the successful development of the technique pinealectomy in the adult monkey. Explant tissues have been established with regular success thus far and apparent evidence of in vitro transformation has occurred. However, problems with monkey procurement have delayed the final portion of the experiment, the return of the cells to the original host. An encouraging result is that detectable levels of hyroxy-indole-orthomethyl transferase levels have been found in the in vitro transformed pineal cells. This enzyme is specific for pineal tissue and indicates that these transformed cells have retained biochemical markers characteristic of their organ of origin.

The cooperative efforts between the Surgery Branch and the Endocrinology Branch continue to be fruitful in the general area of surgical endocrinology. The use of human menopausal gonadotropins in the treatment of anovulatory infertile women continues to fulfill its earlier promise. Both the estrogen reponse and the ovulatory responses have been shown to be dose related. Pre-treatment studies of urinary gonadotropins, urinary steroids and plasma gonadotropins have been found to be of little help in predicting responsiveness. In the patients in which the use of human menopausal gonadotropins induces ovulation and resulting pregnancy, measurement of plasma chorionic gonadotropin levels in very early pregnancy has shown elevated values beginning as early as the 11th day postconception and rising exponentially for the next 10 days. The correlation between the rate of increase in gonadotropin production by early trophoblast and the geometric considerations of increase in mass of trophoblast suggests rather strongly that the changes in plasma gonadotropin levels are linearly related to the changes in trophoblastic mass. In addition to providing new and important information on normal gestational development, these data strongly substantiate the basic assumption in treatment of gestational trophoblastic neoplasm, namely that gonadotropin excretion accurately reflects the mass of functional neoplasm present. Measurement of plasma gonadotropins in patients under study for anovulatory fertility who have received Clomiphene as part of their study has shown that not only does Clomiphene function as an agent for induction of ovulation, but does so by means of an increase in plasma FSH concentration. Hence Clomiphene may be useful as a test for pituitary gonadotrophic function.

The efforts to achieve transplantation and growth of human choriocarcinoma in a subhuman primate have proved successful. Human choriocarcinoma tissue serially propagated in hamster cheek pouches, is transplanted aseptically into the cerebral cortex of the Rhesus monkey. The monkeys progressively develop signs and symptoms of expanding intracranial tumors and die in about three weeks. In all monkeys viable tumor was recovered from the brain at the time of necropsy. Production of human chorionic gonadotropin by the tumors has been demonstrated by both bio-assay and radioimmunoassay. The successful development of this model provides a system that will be useful in studying the growth of human choriocarcinoma in a subhuman primate from both the

endocrinologic and immunologic point of view. In addition the model provides a laboratory preparation in which can be studied all the phenomena of the development of a rapidly expanding intracranial tumor. Angiographic changes, radioisotope scans, development of brain edema and reactions to increasing intracranial pressure have all been studied with this model.

Because further extension of surgical resectional procedures in the treatment of cancer will require organ transplantation, the Surgery Branch continues its active interest in this area. Many, if not all, the technical problems of lung transplantation have been solved in the large animal laboratory. Long-term survivors with normally functioning lungs have been achieved after lung auto-transplantation in five dogs, two monkeys, and one baboon. Following solution of these technical problems, current efforts are being directed toward modification of the homograft rejection response so that long-term lung homotransplantation in animals will be possible.

A method for preparation of water soluble transplantation antigens has been studied, since the availability of such a preparation would greatly facilitate further investigation into the problem of damping the homograft rejection response. Carcinogen-induced guinea pig sarcomas have been subjected to sonication, clarification by centrifugation, gel filtration and finally electrophoresis. The fraction so isolated has been shown to elicit delayed cutaneous hypersensitivity reactions in animals of another strain sensitized by homograft of tumor from which the soluble antigen was obtained and in animals of the same strain sensitized by previous exposure to the same tumor. These data are of considerable interest because it seems that both the transplantation antigens and the tumor specific antigens of these sarcomas have been obtained in a relatively pure state. These results have major importance both in tissue transplantation work and in studies of tumor immunology. Additionally, the demonstration that delayed cutaneous hypersensitivity of a tumor specific nature can be used to assess the state of reactivity of the host against this particular tumor is a major methodological step forward to facilitate study of the mechanisms involved in syngeneic tumor immunity.

Attempts to devise new ways of suppressing the homograft response have progressed during the past year. Phytohemagglutinin has been shown to induce antigen-like responses in lymphocytes *in vitro* and to effect favorably the take of skin allografts *in vivo* in rabbits. Study of skin graft prolongation in dogs has shown that phytohemagglutinin and Azothioprine, a commonly used immunosuppressant, when given in nontoxic doses in combination produced quite effective immunosuppression with prolongation of skin grafts. The effect is far greater with low dose combinations than the effect of higher and more toxic doses of either substance alone. Present efforts are being directed toward elucidation of the mechanism of this immunosuppression using soluble antigens for the evaluation of antibody production under these various immunosuppressive regimens.

A method for the demonstration of humoral antibody to histocompatability factors or humoral antibody to carcinogen-induced tumor has been under development this past year with considerable success. The method involves treating target cells with antisera and then adding a radioisotope labeled antibody to

the mouse immunoglobulin whose presence is being sought. The radioactivity of the indicator globulin can be counted at the conclusion of the experiment, and quantitative results obtained across a continuous spectrum of values rather than the stepwise range obtained with the usage of serial dilution immunological techniques. Reliable detection of humoral antibody to transplantation antigens has been routinely accomplished with this method. Results from studying F_1 hybrid animals and their parental strains suggests that this may be a useful test for determining degree of histocompatibility. Using the same method, tumor specific humoral antibodies are being sought and the results appear promising but are still in very preliminary stages.

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Summary of Consultative Requests Received by NCI Surgery Branch Staff

1963 - 1967

	NCI Patients	Other NIH Institute Patients	Total
1963	366	854	1220
1964	356	690	1046
1965	429	811	1240
1966	362	843	1205
1967	343	789	1132

These numbers refer to individual requests received in our office and answered by personal staff consultation. The actual consultative patient visits are much greater. On an average, the patients is seen 4 times after the original consult for follow-up advice and treatment.

TABLE 2

Individual Intramural Consultations Received by Surgery Branch, NCI

Listed by Institutes

1963 - 1967

	NCI	NINDB	NIAMD	NHI	NIAID	NIMH	NIDR	NICHD	TOTAL
1963	366	184	221	228	158	37	26	-	1220
1964	356	121	207	201	104	33	24	-	1046
1965	429	129	216	277	119	39	31	-	1240
1966	362	271	201	197	86	59	11	7	1205
1967	343	215	245	166	85	61	17	-	1132

TABLE 3

Summary of Extramural Consultations Requested by or through Surgery Branch, NCI

1963 - 1967

	ENT	General and Plastic Surgery	Gynecology	Urology	Oncology	Total
1963	346	14	7	18	9	394
1964	233	6	4	4	3	242
1965	268	6	2	9	1	286
1966	271	8	4	13	2	298
1967	294	11	1	1	1	308

Extramural consultations are obtained from a very cooperative panel covering chiefly the ear, nose and throat specialty. They see patients two afternoons a week consulting chiefly on problems of hearing and sinusitis. When considering the routine, relatively uninteresting type of consult requests these are, I am most appreciative of the service offered by Doctors Moffett, MacFarland, Webb, Fields and Pumphrey in coming to the National Institutes of Health 85 times in the past year and seeing 294 patients. Other surgical consultants are recorded in the Clinical Center records as visiting the National Institutes of Health, but these were requested directly by an individual institute to the consultant.

TABLE 4

Annual Report of NIH Surgical Sites
Listing the Number of Major & Minor Procedures
Carried out by Each of the Operative Units at NIH

Patient		NCI	NHI	NINDB			NIDR	Cons.	TOTAL
				Neuro.	Eye	Muscle			
NCI	Maj.	328	0	15	0	0	2	8	353
	Min.	257	1	2	1	2	1	3	267
NHI	Maj.	15	238	2	0	0	2	5	262
	Min.	16	14	2	0	8	5	1	46
NINDB	Maj.	7	0	76	15	0	0	2	100
	Min.	15	0	14	7	163	0	1	200
NIAMD	Maj.	43	3	3	0	0	1	6	56
	Min.	22	0	0	1	19	0	2	44
NIAID	Maj.	8	3	5	0	0	0	2	18
	Min.	9	1	2	1	2	1	1	17
NIDR	Maj.	0	0	1	0	0	6	0	7
	Min.	2	0	0	0	0	2	0	4
NIHM	Maj.	1	0	0	0	0	0	0	1
	Min.	2	0	1	0	0	0	0	3
Total Cases		725	260	123	25	194	20	31	1378
	Major	402	244	102	15	0	11	23	797
	Minor	323	16	21	10	194	9	8	581

TABLE 5

Total Surgery Performed by NCI Surgery Branch Staff 1967

	10 East	Other NCI	Other Institutes	Total
1963	470	159	399	1028
1964	424	215	256	895
1965	394	112	199	705
1966	410	113	218	741
1967	440	145	140	725

TABLE 6

Total Surgery Performed by NCI Surgery Branch

Listed by Magnitude of Procedure

1967

	10 East	Other NCI	Other Institutes	Total
Major	290	36	47	373
Minor	150	109	93	352
	440	145	140	725

Serial No. NCI-3800

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Removal of Cancer and Other Tissues for Histologic Biochemical and other Studies as Required by Scientists and other Investigators for Correlated Studies.

Previous Serial Number: Same

Principal Investigators: Entire Staff of the Surgery Branch, NCI

Other Investigators: Many members of other Institutes with whom cooperative studies develop.

Cooperating Units: Anesthesiology Department, Clinical Center and all clinical units of the National Institutes of Health.

Man Years: (July 1, 1967 through June 30, 1968)

Total:	4½
Professional:	4
Other:	½

Project Description:

Part I. Service aspects

Part II. Specific cooperative studies
- A. Pheochromocytoma
- B. Hyperparathyroidism
- C. Lymphoma and Hodgkin's Disease
- D. Mechanism of taste and smell
- E. Mechanism of new bone formation in osteoporosis

Project Description:

Part I. Service aspects

Objectives:

The object of this project is to make available human tissue for histologic, histochemical, biochemical, biophysical, viral and immunological studies to scientists working in various branches of the National Cancer Institute and other institutes, who require tissue for studies. As a rule, such studies are

not done as cooperative projects. In addition, it implies furnishing general surgical consultative coverage of patients admitted to other branches of the National Cancer Institute and/or other institutes and assisting with their study projects.

Methods Employed:

Methods employed are standard surgical procedures. Material from this project is obtained mostly from patients hospitalized for study in other branches or institutes. Occasionally it is necessary to admit patients for specific studies in which a specific type of tissue is obtained. Tumor tissue, lymph nodes, muscle, skin and liver comprise the bulk of tissue removed for study.

Major Findings:

In that the major portion of the work involved in this project is done as a surgical consultative service and not as a cooperative study, the project results are not available to the surgical service. During the course of supplying this service 1,132 individual requests for consultation were received. Of these requests 58% resulted in an operative procedure, 33% of which entailed major surgery. These figures do not include the extensive follow-up which we afford each of these consultations but only the original written request to participate in their care.

Significance to Bio-medical Research and Program of the Institute:

This project is a service function for other branches of the National Cancer Institute and National Institutes of Health. It is seldom that the Surgery Branch is included in the research studies other than supplying the tissue to the investigator, however, this trend is being reversed and several pilot studies are being carried out on a cooperative basis.

Proposed Course of Project:

Continued cooperative assistance within the physical limits of the Surgery Branch staff.

Project Description:

Part II. Specific cooperative studies
A. Pheochromocytoma

Objectives:

The goals of this project have been; 1) the development and evaluation of a joint medico-surgical program for the overall management of patients with pheochromocytoma and 2) continuing clinico-pathologic scrutiny of the operative material from these cases in an effort to define more clearly the malignant potential possessed by this unusual tumor which lacks the usual

histologic criteria for malignant in spite of demonstrating its biologically malignant nature in about 10% of cases.

Methods Employed:

Standard surgical and anesthetic procedures are used when operating upon these patients. The analytical methods for catechol amine determinations are those developed and currently employed by the Experimental Therapeutics Branch, NHI. The patients studied are those who have been referred to the Experimental Therapeutics Branch, NHI, for evaluation and treatment of their pheochromocytoma.

Dr. Philip Grimley, of the Pathologic Anatomy Branch, has added the technique of electron microscopy to the usual histopathologic methods used for pathologic studies.

Major Findings:

From our experience of 16 cases thus far, several new concepts of interest have evolved: (1) Attempts at preoperative localization of the site of an intra-abdominal pheochromocytoma may be omitted and the tumor(s) found at operation by observing blood-pressure rises in response to manipulation of possible sites for tumor. (2) Based on the hypothesis that these lesions all possess important neoplastic potential, a more extensive operation than is usually done is believed to be warranted. (3) Two extra-adrenal lesions which produced significant amounts of epinephrine have weakened the dogma that all epinephrine producing pheochromocytomas are intra-adrenal. (4) Surgical reduction of amount of functional tumor may be of benefit in patients with surgically incurable malignant pheochromocytoma.

Significance to Bio-medical Research and Program of the Institute:

There are few tumors which have a dual uniqueness similar to pheochromocytoma, i.e., a functional lesion with an easily measured secretory product but which lacks intrinsic histologic criteria of malignancy. This combination provides a rare opportunity to study the effectiveness of vigorous surgical therapy using as an endpoint something other than the ultimate demise of the patient from disseminated disease.

Proposed Course of Project:

The cooperative study will continue, with a greater emphasis placed upon elucidation of relative degrees of malignancy among the various lesions and upon attempts to benefit more patients with malignant pheochromocytomas by extensive operation.

Project Description:

Part II. Specific cooperative studies

B. Hyperparathyroidism

Objectives:

The Clinical Endocrinology Branch, NHI, and the Section on Mineral Metabolism Metabolic Diseases Branch, NIAMD, have individual programs underway to determine clinical and laboratory criteria which justify surgical exploration of the neck for the treatment of parathyroid disease. This joint medico-surgical program will increase the reliability of diagnosis and efficacy of treatment of these disorders, especially with respect to the problems of renal damage due to secondary urinary stone disease.

Methods Employed:

Standard surgical and anesthetic procedures are used when operating on these patients. In addition to the customary extensive clinical evaluation and laboratory determination of static derangements of calcium and phosphorus metabolism, a battery of tests are performed to measure responses to induced distortions of calcium and phosphorus homeostasis. At operation, prior to any extirpative maneuver, intensive efforts are made to positively identify by frozen section all parathyroids present. Selective inferior thyroid artery angiographic studies are done preoperatively in most cases to aid in identification of the site of the abnormal parathyroid.

Major Findings:

The angiographic method has been of considerable value in localizing adenomatous parathyroids, particularly in several patients who remained clinically and biochemically hyperparathyroid after neck surgery performed elsewhere. Results of surgical ablation of parathyroid tissue in chronic renal stone formers without overt evidence for hyperparathyroidism but with abnormal responses to distortions of calcium homeostasis continue to support the concept that earlier surgery benefits such individuals in that their hyperparathyroidism is ocrrected before extensive renal damage occurs. The reliance on frozen section identification of parathyroids during surgery has resulted in improved precision of operation, so that in the cases explored during the past year, all have been relieved of their hyperparathyroidism while none have been rendered permanently hypoparathyroid.

Significance to Bio-medical Research and Program of the Institute:

The angiographic technique for demonstration of adenomatous parathyroids is sufficiently successful that, although the case material has not yet permitted testing the hypothesis, it is believed that the distinction between benign and malignant parathyroid lesions may be made preoperatively with an improved operative procedure resulting from such information. The importance of the development of a more effective therapy that may be applied earlier in the course of a disease process is self-evident. The extensive laboratory and metabolic studies performed before and after operative progressively

broaden the understanding by all members of the team of the endocrinology involved.

Proposed Course of Project:

This study continues with the full enthusiasm of all cooperating units. The addition of selective venous catheterization of the drainage from the abnormal parathyroid, made possible by arteriographic demonstration of the abnormality, will permit a more accurate diagnosis based on venous blood measurements of parathyroid hormone levels.

Project Description:

Part II. Specific cooperative studies
C. Lymphoma and Hodgkin's Disease

Objectives:

To allow more accurate staging of patients with lymphoma and Hodgkin's disease. At present, radiographic and biochemical studies are generally used to determine the presence or absence of involvement of intra-abdominal and intra-thoracic lymph nodes and viscera. The limitations of these methods of study lead to incorrect staging, but improper treatment, and invalidates related statistical studies of the diseases.

Methods Employed:

All new patients with lymphoma and Hodgkin's disease admitted to the National Institutes of Health for study and treatment are evaluated and staged jointly by the radiation and chemotherapy services. Patients with equivocal or border-line equivocal liver chemistries, lymphangiograms of retroperitoneal lymph nodes, and other studies of the chest and abdomen, are referred to the Surgery Branch for further evaluation and surgical exploration and biopsy if deemed appropriate. Also, patients with questionable recurrences in previously radiation-treated areas are referred for similar evaluation.

Major Findings:

No results of exploration and biopsy of a portion of the patients referred thus far demonstrate hitherto unappreciated limitations of radiographic criteria for the involvement of lymph nodes by lymphoma and Hodgkin's disease. In several patient, biopsies have failed to reveal tumor in lymph nodes which on the basis of their lymphangiographic appearance would have been treated with radiation or chemotherapy, Conversely, in a small group of patients, tumor was found on biopsy of lymph nodes deemed not sufficiently suspicious on lymphangiographic exam to warrant treatment. Biopsies of suspicious lesions in previously treated patients have demonstrated the inadvisability of low-dose "prophylactic" radiation treatment of lymph node

regions because of the high rate of recurrence in these areas in spite of much treatment. Retrospective study of these cases of "radiation failure" has also lead to better criteria for the identification of patients with potentially more resistant or more virulent forms of Hodgkin's disease prior to treatment.

Significance to Bio-medical Research and Program of the Institute:

Valid evaluation of modalities and methods of treatment of lymphoma and Hodgkin's disease hinge on accurate initial staging. New studies are providing additional material for improved criteria of staging.

Proposed Course of Project:

The Surgery Branch will collaborate with the radiation and chemotherapy services in the evaluation of the staging criteria of these diseases on a continued basis.

Project Description:

Part II. Specific cooperative studies
D. Mechanism of taste and smell

Objectives:

To investigate the mechanism of taste and smell.

Methods Employed:

A standard set of physiologic stimulants are used in patients before and after major surgery in which an anatomical area dealing with one of their functions is removed. The importance of this area in the process of taste or smell can then be determined. These studies are combined with light and electron microscopic studies of individual taste papillae.

Major Findings:

In many patients in whom all of the olfactory epithelium and olfactory nerves have been removed, some ability to smell still persists. This ability has been located in secondary centers of olfaction located in the pharynx and larynx.

In patients who have had the larynx removed, their ability to smell is sharply decreased. The reason for this has not been identified.

Significance to Bio-medical Research and Program of the Institute:

Mechanisms involved in taste and smell are complex and poorly understood. This type of study will add additional information which has not previously been obtained.

Proposed Course of Project:

Additional patients are necessary in order to have statistically significant figures.

Project Description:

Part II. Specific cooperative studies
E. Mechanism of new bone formation in osteoporosis

Objectives:

To establish the effectiveness of various treatment schedules in producing new bone formation in patients with osteoporosis.

Methods Employed:

The administration of tetracycline for seven days to patients results in the labeling of osteoclasts so that they can be identified with fluorescent microscopy. By performing the laveling process at two time intervals 8-10 weeks apart, the rate of new bone formation can be determined by measuring the distance between the two fluorescent lines. To do this, a full thickness rib biopsy is obtained under local anesthesia after the labeling process.

Major Findings:

The labeling process and the rib sections employed have been sufficient to identify the two fluorescent lines and measure the rate of new bone formation. The number of patients entered into the study to date are too few to give any statistically significant results.

Significance to Bio-medical Research and Program of the Institute:

The method for the first time gives the investigator a good objective method of assessing in a short time interval the effectiveness or lack of effectiveness of the treatment regimen employed. It may be possible to sub-classify the disease osteoporosis into various sub-categories depending upon their response to various treatment regimens.

Proposed Course of Project:

Additional patients are necessary and will be added to the study as the preliminary results are evaluated.

TABLE 1

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1963 - 1967

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TABLE 2

Individual Intramural Consultations Received by Surgery Branch, NCI

Listed by Institutes

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1966	362	271	201	197	86	59	11	7	1205
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TABLE 3

Summary of Extramural Consultations Requested by or through Surgery Branch, NCI

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Extramural consultations are obtained from a very cooperative panel covering chiefly the ear, nose and throat specialty. They see patients two afternoons a week consulting chiefly on problems of hearing and sinusitis. When considering the routine, relatively uninteresting type of consult requests these are, I am most appreciative of the service offered by Doctors Moffett, MacFarland, Webb, Fields and Pumphrey in coming to the National Institutes of Health 85 times in the past year and seeing 294 patients. Other surgical consultants are recorded in the Clinical Center records as visiting the National Institutes of Health, but these were requested directly by an individual institute to the consultant.

TABLE 4

Annual Report of NIH Surgical Suites
Listing the Number of Major & Minor Procedures
Carried out by Each of the Operative Units at NIH

Patient		NCI	NHI	NINDB			NIDR	Cons.	TOTAL
				Neuro.	Eye	Muscle			
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	Min.	16	14	2	0	8	5	1	46
NINDB	Maj.	7	0	76	15	0	0	2	100
	Min.	15	0	14	7	163	0	1	200
NIAMD	Maj.	43	3	3	0	0	1	6	56
	Min.	22	0	0	1	19	0	2	44
NIAID	Maj.	8	3	5	0	0	0	2	18
	Min.	9	1	2	1	2	1	1	17
NIDR	Maj.	0	0	1	0	0	6	0	7
	Min.	2	0	0	0	0	2	0	4
NIHM	Maj.	1	0	0	0	0	0	0	1
	Min.	2	0	1	0	0	0	0	3
Total Cases		725	260	123	25	194	20	31	1378
	Major	402	244	102	15	0	11	23	797
	Minor	323	16	21	10	194	9	8	581

TABLE 5

Total Surgery Performed by NCI Surgery Branch Staff 1967

	10 East	Other NCI	Other Institutes	Total
1963	470	159	399	1028
1964	424	215	256	895
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1966	410	113	218	741
1967	440	145	140	725

TABLE 6

Total Surgery Performed by NCI Surgery Branch

Listed by Magnitude of Procedure

1967

	10 East	Other NCI	Other Institutes	Total
Major	290	36	47	373
Minor	150	109	93	352
	440	145	140	725

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Davis, E.E., Newsome, H.H., Wright, L.O., Hammond, W.G., Easton, J. and Bartter, F.C.: Bilateral adrenal hyperplasia as a cause of preliminary aldosteronism with hypertension, hypocalcemia and suppressed renin activity. Amer. J. of Med. 42: 642-647, April 1967.

Doppman, J., Foley, H.T. and Hammond, W.G.: Inferior vena caval compression: The pseudo-obstruction syndrome. Amer. J. of Roentgen. 100: 411-416, June 1967.

Engelman, K. and Hammond, W.G.: Adrenaline production by an intra-thoracic pheochromocytoma. Lancet, I: 609-611, March 1968.

Morton, D.L., Erhenfeld, W. and Wylie, E.J.: Significance of outflow obstruction following femoral popliteal thromboenarterectomy. Arch. Surg. 94: 592-599, May 1967.

ARTICLE IN PRESS:

Pilch, Y.H.: A book review of "Hamilton Bailey's demonstrations of physical signs in clinical surgery." (The New Physician)

Serial No. NCI-3801

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Evaluation of Cancer Surgery as to its Effectiveness and Safety in Providing Definitive and Therapeutic Care of Cancer Patients

Previous Serial Number: Same

Principal Investigator: Dr. Alfred S. Ketcham and Entire Staff, Surgery Branch, NCI

Other Investigators: Dr. John L. Doppman and Dr. William L. Ashburn

Cooperating Units: Diagnostic Radiology Department, Pathologic Anatomy Department, Clinical Pathology Department, Nursing Department, Anesthesiology Department, Social Work Department, Rehabilitation Department, Clinical Center; NIDR, Applied Mathematical and Statistics Section, Biometry Branch, NCI, Members of the Endometrial Carcinoma Cooperative Study Group

Man Years: (July 1, 1967 through June 30, 1968)

Total:	5-1/2
Professional:	5
Other:	1/2

Project Description:

Part I. Study of the biologic behavior and results of surgical therapy of cancer of the uterine cervix

Part II. Study of the biological behavior and results of surgical therapy of cancer of the head and neck regions

Part III. The role of bacterial infection in cancer surgery

Part IV. A study of multiple primary cancers

Part V. The evaluation of uretero-ileostomy in urinary diversion

Part VI. Selective pancreatic (celiac) arteriography

Part VII. Pancreatic scanning using toluidine blue

Part VIII. Reflux pancreatography

Part IX. A comparison of surgery and combined surgery-radiotherapy in the treatment of endometrial adenocarcinoma

Project Description:

Part I. Study of the biologic behavior and results of surgical therapy of cancer of the uterine cervix

Objectives:

1. To record the natural or unnatural history of this type of cancer and the effects of surgical extirpation
2. To develop new methods of salvaging the radiation failure cases
3. To demonstrate the results of pelvic exenterative surgery in this type of disease

Methods Employed:

Indications for surgical therapy include extensive localized disease without tumor dissemination beyond the anatomical pelvis without pelvic vessel obstruction and without sciatic plexus invasion. No definitive pelvic, surgical exploration was carried out if preoperative or operative findings indicated complete tumor removal was not feasible. The scope of operative procedures has ranged from simple hysterectomy to total pelvic exenteration. Approximately 45 patients a year are seen with advanced or radiation recurrent carcinoma of the cervix. Over the past ten years, 52% of the referrals have been radiation recurrent and within the past year, 61% were failures to previous therapy. Thirty patients in the past year underwent exploration to determine the feasibility of cure by surgery, 20 of which received definitive surgery.

Major Findings:

The cumulative five-year survival following radical hysterectomy is 72%, anterior exenteration at 32% and total pelvic exenteration at 29%. Hospital mortality is 8% for the total pelvic exenteration group, 4% for anterior exenteration and 1% for radical hysterectomy.

Significance to Bio-medical Research and Program of the Institute:

By a rather aggressive approach to the problem of what is so often deemed incurable cancer of the cervix, the surgeon can offer a significant survival with procedures acceptable to the patient, referring physicians and family, and can return many patients to life as socially acceptable individuals often in a wage-earning capacity. The extension of hemipelvectomy as a one staged procedure with total pelvic exenteration in selected individuals has been carried our in 4 patients with carcinomas of the cervix with lateral pelvic wall fixation. There has been no mortality and three of these ladies

are free of disease at 64, 22, and 21 months. These severely traumatized patients afford our laboratory of surgical physiology a unique source of study material.

Proposed Course of Project:

While continuing to review the indications for surgery and to investigate the reasons for failure even by radical surgery, we are trying to determine more specific and reliable criteria for operability and curability without extending the scope of surgical dissection. It has definitely been shown that unilateral leg edema and sciatic distribution of leg pain are reliable signs of incurability. Lymphangiography has become a research tool used to direct the surgeon's attention to possible areas of lymph node metastasis. In addition, the inadvisability of posterior pelvic exenteration as treatment for cervical cancer has been shown.

Project Description:

Part II. Study of the biological behavior and results of surgical therapy of cancer of the head and neck regions

Objectives:

1. To determine the feasibility of an aggressive surgical approach to irradiation or surgical recurrent malignancies of the head and neck areas

2. An evaluation of adjunctive immediate preoperative irradiation in previously untreated cancer of this anatomical area

3. To determine the effectiveness of a combined intra-cranial facial approach to cancer of the paranasal sinuses

4. To determine the prevalence, site of origin, and rationale of treatment of unknown primary disease, metastatic to head and neck areas

5. The clinical pathological correlation of epidermoid carcinoma of the head and neck area

6. To determine the significance of tumor cells in the washings of the wound at surgery and their presence in wound drainage, as correlated in follow-up clinical-pathological studies

Methods Employed:

Following extensive preoperative clinical, pathological and roentgenological studies standard radical surgical procedures are carried out. (a) In patients having previously untreated malignancies in the head and neck area, preoperative irradiation has been given to the local area of disease in the

amount of 1,000 roentgen 24 hours prior to surgery. (b) Patients having paranasal sinus cancer, either primary or recurrent, which suggest roentgenographically or clinically that the cribriform plate will have to be removed at the time of surgery are approached from an intra-cranial and combined facial surgical incision. This permits en bloc removal of the tumor at the same time affording protection of the surrounding vital structures. (c) In patients requiring surgery to control recurrent disease following previous therapy. During the continuing study, 80% of the patients screened for admission have been failures to previous therapy, usually radiation. There is an alarming increase in the number of referrals now received following failure of chemotherapy. The actual incidence of patients admitted and treated, who have been previous failures is 71%, a very distressing commentary upon the general misunderstanding of head and neck malignancies in the referring community.

Major Findings:

The appearance of recurrent local disease, if treatment has not been successful, is noted within 18 months in over 50% of the incidences and within 6 months in 25% of the incidences. There has been a significant correlation between local recurrence and failure of further treatment. The patient who develops rapidly recurrent malignant growth in the operative field, can be offered little survival by further treatment. There has been no correlation between local recurrence and distance spread and a 51% incidence of the distance metastasis following what is thought to be definitive surgery. Seventy-three patients have been included in the preoperative irradiation study. The concept of a small dose of irradiation preventing tumor dissemination of actively viable cells at surgery is based upon animal studies carried on in this institute and has been underway clinically for the past five years. Preliminary review of the series indicates that there may be little effect obtained by this dose of preoperative irradiation in decreasing the incidence of wound recurrence following surgery and the study has been halted while an evaluation is made of the results.

The combined intra-cranial facial operation for cancer of the paranasal sinuses has been performed in 34 patients to date. A review of the results as of April, 1967 revealed that of 34 patients treated, 14 were dead and 20 were living for a time interval of eight months to ten years follow-up. The operation has been extended to include patients showing some involvement of the cerebral meninges. All of the patients which have thus far been approached in this manner, have had extensive disease and have not been amenable to treatment in any other fashion. Any survival at all is indeed promising, but more patients and more time is necessary before the procedure can be recommended for routine use. The advantages of the combined procedure are that it involves (1) evaluation of intra-cranial disease, (2) protection of the brain, (3) avoidance of spinal fluid fistulas, (4) more adequate hemostasis, (5) facilitation of resection, (6) en bloc resection.

Unknown primary lesions with neck metastases is not a frequent occurrence

and accumulation of a series of significant numbers is difficult. The eventual discovery of the primary lesion has usually been in the nasopharyngeal sinus area but three cases have been from the thyroid and one from the kidney. In the past year, five new cases have been admitted to this study.

Previous studies of the presence of tumor cells in operative wound washings revealed an incidence of 26% positive cells. No significant correlation could be drawn between the presence or absence of cells and the incidence of recurrent disease or survival. In the past year the incidence of "positive" washings has dropped to 11% and in light of this the earlier data is being reviewed. It is felt that the lower incidence of positive cells in the past year is due to more stringent cytological criteria for the reading of a positive cell.

Significance to Bio-medical Research and Program of the Institute:

These locally resistant, usually recurrent head and neck lesions have long been a problem to oncologists. We are attempting to correlate agressive surgical exploration of these lesions with survival and the mode of tumor dissemination. Careful environmental background reviews suggests a close relation between the Surgery Branch and the Dental Prosthetic Unit is allowing a comprehensive evaluation of the indications for plastic reconstruction of facial deformities and evaluation of newer prosthetic materials.

Proposed Course of Project:

The extent of surgical excision cannot be much increased in its scope beyond that which we are now able to perform successfully. More patients are necessary before the combined cranial-facial approach to paranasal sinus cancer can be recommended for routine use. Both procedures are based on sound experimental and clinical observations and final evaluation should prove rewarding. Pilot studies are underway to determine the feasibility of intensive preoperative medication in patients with disease which has locally extended beyond what can be cured by conventional means.

Project Description:

Part III. The role of bacterial infection in cancer surgery

Objectives:

To further substantiate findings in the previous years of the evaluation of the operative wound infection rate and its relation to the "prophylactic" use of antibiotics following extensive cancer surgery.

Methods Employed:

By immediate admission and repeated preoperative culturing of all patient's nose, skin and tumor, patients are classified into either carrier or non-

carrier status of hemolytic staphylococcal aureus coagulase positive organisms. By bacteriophage typing, through the cooperation of the Clinical Pathology Department, a correlation could be drawn between the patient's preoperatively carried organism and that carried postoperatively, either asymptomatically or related to a postoperative infection. In previously reported studies patients undergoing radical cancer surgery had, on a double-blind basis, either ten days of Chloromycetin postoperatively, or a placebo. A subsequent study entailed the use of seven days of Chloromycetin postoperatively in all patients, while on a double-blind basis, the patients received preoperatively for ten days, either three days of placebo or three days of antibiotic. The current protocol utilized intravenous antibiotics before, during and seven days following the operation. Following two separate studies, which have been reported, antibiotic therapy has been used prophylactically in a therapeutic manner on all patients undergoing cancer therapy. These patients have been carefully observed for wound infection and any possible correlation to their "carrier status" or pathogenic organisms.

Major Findings:

The previous annual reports detail the specific results of both double-blind antibiotic studies. In brief, the infectious complication rate following extensive cancer surgery was lowered from 54% to 14% by the use of pre- and postoperative antibiotics rather than placebo. A 95% infection rate occurred when the intraoperative use of antibiotics was employed.

Table 5 details the results for the last 12 years. The 1960 rate was elevated to 32% in that this was the year of the original study, whereas 1959 was lowered somewhat because of the discontinuance of the use of penicillin and streptomycin and the selection of antibiotics was left to the discretion of the surgeon.

TABLE 5
11 YEAR SUMMARY OF INFECTION RATES AND
STAPHYLOCOCCAL INCIDENCE FOLLOWING EXTENSIVE CANCER SURGERY

Year	Extensive cancer operations	Infection rate per cent	Per cent of total infections that were staphylococcal
1956.....	128	28	35
1957.....	139	31	42
1958.....	145	30	59
1959.....	130	22	69
1960.....	144	32	74
1961.....	115	13	73
1962.....	132	10	72
1963.....	137	15	70
1964.....	131	7	51
1965.....	132	6	36
1966.....	141	5	22
1967.....	128	6	17

During the past 11 years, 1676 extensive cancer operations have been performed on the Surgery Branch of the National Cancer Institute. During the early part of this study the infectious complications ranged from 22% to 31% during a time when penicillin and streptomycin were used somewhat routinely at the discretion of the surgeon. The results of a double-blind antibiotic-placebo study revealed an infection rate of 54% when a placebo was used and 14% when routine ten-day postoperative antibiotic therapy was instituted. A subsequent double-blind study showed that by giving drug for three days preoperatively and seven days postoperatively, the infection rate was 8.8%; whereas, preoperative placebo and seven days postoperative antibiotic therapy resulted in a 17% rate of infectious complications. With continued use of antibiotic prophylaxis, while continuing the same type of extended operative procedures, the infection rate was 10% and 15%. Chloramphenicol, 2 gm. every six hours, was the antibiotic used and there were no significant complications referable to drug toxicity and only five severe infectious complications referable to loss of staphylococcal sensitivity to the drug.

Over the past years the percentage of total infections that were hemolytic staphylococcal aureus DNase positive in origin progressed from 35% in 1956 to 70-75% in 1961-1964, and dropped to 36% in 1965 and 17% in 1967. At the same time the incidence of patients who were carriers of staphylococci on admission to the hospital was 32% and has remained at the approximate level. Patients who carry staphylococci into the operating room, usually asymptomatically, run at 28% increased rate of infection over non-carriers. Ninety-three per cent of these patients will become infected with the same phage typed organism that they carried into the operating room. This suggests that

self-infection may play as an important a role in infectious complications as does cross contamination.

During the past three years, 12 to 24 hours of preoperative antibiotics have been used routinely along with continuous intraoperative antibiotic drugs and three to seven days of postoperative therapy. Table 6 lists the over-all infectious experiences of patients undergoing very radical cancer surgery.

TABLE 6
POSTOPERATIVE INFECTIONS COMPLICATION RATE
FOLLOWING DIFFERENT MODES OF ANTIBIOTIC THERAPY

	# of Pts.	Infections	Rate
Antibiotic used at discretion of surgeon	704	197	28%
No antibiotic therapy	55	28	54%
Postoperative therapy	255	43	17%
Pre & postoperative therapy	215	23	11%
Pre, post and intraoperative therapy	432	26	6%

Significance to Bio-medical Research and Program of the Institute:

When postoperative morbidity and mortality are increased due to complications related not directly to the cancer but due to infection, the over-all evaluation of surgery and its place in cancer therapy is markedly altered. Bringing an alarming infection rate under relative control has decreased morbidity and mortality as indicated in the previous paragraphs. In a hospital such as this where rather elaborate facilities are available for close patient surveillance, studies such as this can be done which can be of significant value to the practicing surgeon.

Proposed Course of Project:

The indications for "prophylactic" antibiotic usage when carrying out the present clinical investigative studies within the Surgery Branch are unequivocable. Close surveillance of carrier patients and personal carriers within the hospital is being continued. It is anticipated that continuation of the surveillance program will not only lessen the so-called epidemic infectious outbreaks occasionally seen on all surgical wards but will allow more rapid and adequate control when such does occur. It is proposed that the critical period of antibiotic coverage is at the time of the operative

procedure, itself and that constant infusion and drug level maintenance of antibiotics during this period is mandatory in cancer surgery.

Project Description:

Part IV. A study of multiple primary cancers

Objectives:

The occurrence of more than one primary carcinoma in the same individual may no longer be considered a medical curiosity. Our attention has been directed toward a number of individuals who have been treated in the Surgery Branch, and who have developed more than one malignant primary. The clinical records of 18 such individuals seen since 1960 have been reviewed.

Because of the small number of cases, our purpose was not epidemiologically oriented; i.e., to investigate relative incidence of multiple lesions, nor were we primarily concerned with the problem of increased susceptibility of those already experiencing cancer to the development of further neoplasms. Rather, we were interested in this problem from the standpoint of clinical detection, since several new primaries were uncovered during clinical evaluation and routine follow-up. It was felt that such information might be of value to the physicians in an area from which a large number of our patients are referred.

Methods Employed:

The complete clinical records of 18 patients seen and treated at the National Cancer Institute were reviewed. These patients had experienced more than one primary malignant disease, and in one instance, five separate neoplasms. Criteria for inclusion in the study were those first set down by Theodore Billroth in 1889, and later revised by Warren and Gates: 1.) Each tumor must exhibit definite histologic criteria for malignancy (in situ lesions discarded) 2.) Each primary must be a distinct entity 3.) The likelihood that one lesion is a metastasis from the other must be excluded.

Major Findings:

Fourteen of the eighteen patients had experienced double malignancies, and three exhibited three separate neoplasms. The remaining patients had been treated for three multicentric lesions of the skin as well as two unrelated carcinomas of the parotid and prostate glands. The patients were then divided into two categories; namely, those who had experienced more than one lesion in related tissue or organ systems (seven patients) and those whose primaries were unrelated (eleven patients). Four of the eighteen patients had more than one cancer in estrogen-sensitive organs (i.e., breast, cervix, corpus uteri and vagina). No evidence, however, has ever proven an endocrinologic basis for multiple malignancies, and our chance of seeing multiple disease in this organ system is heightened by our specific interest in malignancies of the pelvis.

No effort was made to determine the incidence of multiple malignancies in the entire series of patients seen in the Surgery Branch. Our sample is not appropriate for this since it is made up of referrals to a center specializing in recurrent disease and unusual management problems.

The average age of the patients in our series was no different than might be expected in any cancer population. Mean age for the appearance of the first primary was 53 years, and for the second, 57 years. Several clinically significant features pertaining to the detection of multiple primaries were noted. A number of patients entered the hospital with a symptom complex which had been believed to be caused by one obvious primary, but subsequent evaluation uncovered the second lesion, thus altering the method of treatment. A number of patients were operated upon and second preimaries were unexpectedly found at the time of exploration. Several patients displayed evidence of further unrelated malignant disease during routine follow-up examination either in our own out-patient department or in the office of their private referring physician.

Most noteworthy, however, was the fact that ten of the eighteen patients with multiple malignancies were alive and free of disease one to eleven years after treatment. This lended considerable support to our policy for the treatment of multiple primaries; namely, that if the first neoplasm has been satisfactorily controlled, subsequent primaries should be treated with the same aggressiveness as if each occurred alone.

Significance to Bio-medical Research and Program of the Institute:

This data provided us with considerable insight regarding the proper management and outcome of patients with multiple cancers, as well as point out how detection might be facilitated.

Proposed Course of Project:

Since we are highly dependent on follow-up examinations of patients treated here by the referring physician, dissemination of guiding principles in the care of victims of multiple cancer is of obvious value to our own patients. The published report contained a review of the recent literature regarding the subject of multiple primaries.

Project Description:

Part V. The evaluation of uretero-ileostomy in urinary diversion

Objectives:

A continuing study of the physiology and bacteriology of the ileal conduit is being carried out in the Surgery Branch. Evaluation of function and infection of ileal urinary conduits has been carried out on patients whose urinary stream has been diverted during radical surgery for pelvic malignancy.

Patients with non-visualization of one kidney due to ureteral obstruction by pelvic malignancy are being evaluated before and after ileal diversion by excretory urography and isotope renography.

Major Findings:

We have found that persistent and significant upper urinary tract infection occurs more frequently in patients whose conduits show weak peristalsis and who carry residual urine greater then 15 ml. Obstruction of the conduit by constructions and sharp angulation also prolong the emptying rate and increase the incidence of urinary infection.

Kidneys which do not function because of ureteral obstruction usually regain some function on excretory urography after urinary diversion. However, the radioisotope renogram has shown that tubular function often remains very depressed, despite the appearance of adequate function on the IVP. We continue to divert such obstructed kidneys to preserve as much renal function as possible, regardless of duration of obstruction. However, this study has emphasized the importance of following carefully the contralateral normal kidney which carries most of the burden of total renal function in those patients whose previously obstructed kidney fails to regain adequate tubular function after obstruction is relieved.

Significance to Bio-medical Research and Program of the Institute:

All aspects of urinary diversion after radical pelvic surgery continued to concern us, because the most common non-malignant cause of death after recovery from pelvic exenteration is due to renal obstruction or infection.

Proposed Course of Project:

Continued clinical evaluation.

Project Description:

Part VI. Selective pancreatic (celiac) arteriography

Objectives:

To develop a technique for pancreatic visualization suitable for diagnosis of pancreatic tumors by selective arteriography.

Methods Employed:

Large doses of secretin, pancreozymin and cholecystokinin were given intravenously to dogs and monkeys. Following this, selective celiac artery catheterization was done, contrast material was injected, and numerous x-ray films were taken, using the Sanchez-Perez machine.

Major Findings:

It was postulated that intensive pancreatic hormonal stimulation would result in sufficiently augmented pancreatic blood flow to permit glandular opacification by selective arteriography. This was shown not to be the case. Although, the pancreatic blood vessels, particularly the superior and inferior pancreatio-duodenal arteries, became larger (dilated) after hormonal stimulation, glandular opacification did not occur. Hence, this technique is not especially valuable in detection of pancreatic masses.

Significance to Bio-medical Research and Program of the Institute:

None

Proposed Course of Project:

Terminated

Project Description:

Part VII. Pancreatic scanning using toluidine blue

Objectives:

To develop a better pancreatic scanning technique for preoperative detection of pancreatic masses.

Methods Employed:

Toluidine blue is known to stain pancreatic tissue when given intravenously. It was postulated that this effect could be augmented if the dye were given into the arterial supply of the pancreas by celiac artery injection. A suitable isotope of toluidine blue might then permit improved pancreatic scanning. Toluidine blue, tagged with I^{131}, was hence injected into the celiac artery of monkeys and abdominal scans obtained.

Major Findings:

Non-isotope toluidine blue injected into the celiac artery of monkeys, stains the gland a striking deep blue color. The liver and stomach were stained to a much less degree, and this cleared rapidly. When isotopic (I^{131}) toluidine blue was injected and scans obtained, it was evident that color alone was not a reliable index of the distribution of the dye, since both hepatograms and pancreatograms were obtained. Tissue assay revealed equal amounts of radioactivity per gram of wet weight in liver and pancreas. Apparently, the dye had been converted to the leuco base by the liver, thus accounting for the misleading color differential. The overlay of the radioactivity of the liver thus prevents this technique from being superior to the current seleno-merthionine scan, although pancreatic scans were obtained.

Significance to Bio-medical Research and Program of the Institute:

None

Proposed Course of Project:

Terminated

Project Description:

Part VIII. Reflux pancreatography

Objectives:

To develop a technique for pancreatic visualization for diagnostic purposes by transampullary reflux of radiopaque contrast material.

Methods Employed:

The duodenal loop of monkeys were isolated and distended with radiopaque contrast material. The monkeys were then given large doses of secretin, pancreozymin and cholecystokinin to "relax" the sphincter of Oddi. The abdomen was then fluoroscoped frequently to determine the presence of transampullary reflux.

Major Findings:

In about 90% of the cases reflux occurred and excellent visualization of the pancreatic ducts and biliary tree was obtained. An unexpected finding was the presence of pancreatitis in all of the refluxing monkeys.

Significance to Bio-medical Research and Program of the Institute:

This technique may be applicable to human beings for use in the detection of pancreatic masses. It may also have use in evaluating the patient with obstructive jaundice. Modifications of this technique give a laboratory model for the study of pancreatitis in primates.

Proposed Course of Project:

The principal objections to this technique as a clinical tool currently are the unpredictability and the production of pancreatitis. Study is continuing in an attempt to find a means to permit low pressure, controlled reflux.

Project Description:

Part IX. A comparison of surgery and combined surgery-radiotherapy in the treatment of endometrial adenocarcinoma

Objectives:

1. To evaluate in a statistically sound manner, the comparative therapeutic effectiveness of surgery and combined surgery-radiotherapy in patients with localized endometrial adeno-carcinoma.
2. To evaluate the relative effectiveness of internal (radium) versus external (x-rays) preoperative uterine radiation.
3. To study the natural spread of this disease in the uterus and surrounding structures by means of a uniform and intensive study of the surgical specimen.
4. To classify in detail, localized endometrial carcinoma in surgical candidates using mathematical analysis of all information, clinical and pathological, which can be shown to influence prognosis.

Methods Employed:

A cooperative study group had been formed consisting of 40 universities and hospitals, which have agreed to pool their patient material and to follow treatment protocols approved by the study group. The protocol provides for the random assignment of patients into one of three following treatment groups, (a) hysterectomy, (b) hysterectomy with subsequent vaginal irradiation or (c) vaginal and uterine irradiation with subsequent hysterectomy. Detailed pathological examination of the excised specimen will be made. All data will be handled by means of automatic data processing. Follow-up will continue for five years. Statistical analysis of recurrence and survival data will first compare the treatment effectiveness between the three large treatment groups. Subsequent analysis will subdivide the patients into risk groups as determined by certain prognostic factors, and these smaller and more homogeneous groups will then be re-analyzed.

Major Findings:

There are no major findings to date due to the inability to obtain final approval of a randomized therapeutic trial.

Significance to Bio-medical Research and Program of the Institute:

This study, when finally approved and activated, will be the first prospective, statistically valid, controlled study of primary therapy for endometrial carcinoma. It is hoped to provide an answer to a problem which has been recognized for over 35 years.

Proposed Course of Project:

The study group has been formed, the protocol written and approved, and the study approved by the National Cancer Institute and the Directors of the National Institutes of Health. The problem of informed patient consent, as it applies to controlled randomized therapeutic trials has now been

approved by the Surgeon General. With resolution of this problem, activation of the study will take place and patient accession can begin.

Honors and Awards:

None

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Doppman, J. and Johnson, R.: Reflux pancreatography--an experimental study in monkeys. Invest. Radio. 2: 113-118, March-April 1967.

Johnson, R.H. and Doppman, J.: Duodenal reflux and the etiology of pancreatitis. Surgery 62: 462-467, Sept 1967.

Orme, S.K., Chambers, R.W. and Johnson, R.H.: Bilateral adenocarcinoma of the breast following x-irradiation for pubertal gynecomastia. J. Amer. Med. Assoc. 201: 707-708, Aug. 1967.

Ross, W., Johnson, R.H. and Hayes, R.L.: Examination of the mouth. Gen. Pract. XXXVI: 78-86, Aug. 1967.

Weigensberg, I.J., Marshall, J.R. and Powers, W.E.: Radiolucent intracavitary radiation applicators: Their special usefulness in computer calculated dosimetry. Amer. J. Roent. 99: 881-885, April 1967

ARTICLES IN PRESS:

Ketcham, A.S.: Rational for the choice of the surgical procedures (Clinical Center Monograph).

Ketcham, A.S.: The role of surgery in advanced cancer. (U.S. Med).

Ketcham, A.S., Johnson, R.H., Chretien, P.B. and Farmer, J.C.: Surgery in the role of treatment of advanced oral pharyngeal cancer. (Ellis Fischell Cancer Hospital Monograph).

Van Buren, J.M., Ommaya, A.K. and Ketcham, A.S.: Ten years experience with radical combined craniofacial resection of malignant tumors of the paranasal sinuses. (J. Neurol.).

Serial No. NCI-3803

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of Tumor Emboli in Relation to Local Recurrence and Distant Metastases in Human Cancer

Previous Serial Number: Same

Principal Investigators: Dr. John T. West and Roxane Hume

Other Investigators: Drs. Richard A. Malmgren and Elizabeth W. Chu

Cooperating Units: U.S. Public Health Service Hospital, Seattle, Washington and Pathologic Anatomy Branch, NCI

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1/2
Professional:	1/2
Other:	0

Project Description:

Review and evaluation of published works on cancer cells in human blood from 1955 to present.

Objectives:

To determine significance of tumor cells in circulating blood.

Methods Employed:

1. Comparison of techniques of isolating tumor cells from blood.

   (a) Chemical lysis of non-neoplastic cells
   (b) Chemical lysis of non-neoplastic cells plus filtration
   (c) Enzymatic lysis of non-neoplastic cells plus filtration
   (d) Accelerated sedimentation of red blood cells
   (e) Flotation methods
   (f) Differential centrifugation
   (g) Magnetic separation of leukocytes
   (h) Direct filtration

2. Seven factors that effect percentages of patients reported as "Positive" for cancer cells in the blood.

   (a) Variation in number and size of samples
   (b) Site from which blood is collected
   (c) Time at which samples are collected
   (d) Selection of patient for study (degree of differentiation of tumor, tumor type and extent of cancer)
   (e) Efficiency of technique for recovery of cancer cells
   (f) Variation in skill and experience of the cytotechnician
   (g) Objectivity and competence of cytologist in evaluating cells found.

3. Confusion exists between tumor cells and many other large or abnormal cells seen fairly commonly in concentrates of cellular elements of blood.

4. Embolization is not tantamount to metastases due to several factors.

   (a) Non-viability of cells
   (b) Arrest of cells at some site in the vascular system
   (c) Selective endothelial bonding
   (d) Degenerative changes in tumor cells
   (e) Tumor cells must get out of blood vessels in order to metastasize.
   (f) Hyperthermia, irradiation, altered oxygen tension, altered hormonal environment, induction of an immune state to tumor cells, administration of anti-tumor chemotherapeutic agents affect the likelihood of survival of tumor cells in the emboli state.
   (g) Intravascular proliferation of cells emboli from solid tumors is probably quite limited.

5. Relationship of tumor embolization to metastasis.

   (a) Consideration of available reports leads one to appreciate that there is a positive relationship between occurrence of cancer cells in the blood and subsequent development of metastases.
   (b) In animal experimentation the longer the animal is exposed to tumor embolization or the more tumor emboli the animal was subjected to, the greater the likelihood of metastases. Similar factors could be operative with human cancer.
   (c) In some cases showers of tumor cells during operation seem to be casually related to appearance of uniform size metastases shortly thereafter. In other cases, embolization of a large number of tumor cells at the time of surgery did not produce metastases for as long as five years later. One need not be so single minded about the risk of embolization that usual

diagnostic and therapeutic procedures cannot be carried out.

6. Clinical uses of studies of circulating cancer cells in humans.

   (a) The present state of the techniques of studying tumor cell embolization in humans and the state of our knowledge of the biology of cancer do not permit the use of these studies as a reliable aid to diagnosis of cancer, the establishment of prognosis, the selection of therapy or the judging of the effectiveness of cancer therapy.

7. Applicability in the clinical laboratory.

   (a) Improvement in presently available techniques may result in use of studies of cancer cells in the blood in the clinical laboratory.
   (b) Present techniques are expensive and time consuming.
   (c) We must learn the significance of other rare, unusual or abnormal cells in the blood.

Significance to Bio-medical Research and Program of the Institute:

If knowledge of the field of circulating cancer cells is to advance the development of improved methods will be required. This may necessitate development of new equipment and material or significant improvement of those presently available. The physical, biochemical and immunologic features of cancer cells might be used to distinguish them from non-neoplastic cells. Any difference in size, shape, specific gravity, behavior or chemistry between a cancer cell and a non-cancer cell could be used to help segregate the cancer cells so that they could be quantitated and studied. Study of the hosts ability to dispose of emboli cancer cells involves so many factors that the opportunities for research in this area seem almost unlimited. Such factors that might be relevant include; natural and induced cytotoxicity, mediated by humoral or immunologic mechanisms, phagocytosis, inflammation, and anti-inflammatory substances, coagulability of blood, fibroplasia, hemodynamincs and others. It is possible that new methods may open the door to avenues of investigation not directly related to the hematogenous spread of cancer and perhaps not related to cancer at all.

Proposed Course of Project:

There is no present plan for continuing the study of circulating tumor cells.

Publications:

ARTICLE IN A BOOK, PAMPHLET, OR BULLETIN:

West, J.T. and Hume, R.: Tumor embolization in Humans. Studies of cancer cells in the blood. Progress in Clinical Pathology Chapter I, 1967. New York, New York, Grune & Stratton. (In Press)

Serial No. NCI-3804

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Evaluation of the Nature of Preventing Seeding of Operative Wounds Using Transplantable Animal Tumors.

Previous Serial Number: Same

Principal Investigators: Drs. Alfred S. Ketcham, Robert C. Hoye and William G. Hammond

Other Investigators: Drs. Yosef H. Pilch, Donald L. Morton, James J. Ryan, John D. Harrah, David F. Paulson, Frank C. Sparks, Jean B. deKernion and Richard S. Myers, Paul B. Chretien

Cooperating Units: None

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1 1/2
Professional:	1
Other:	1/2

Project Description:

Part I. Significance of tumor cells in operative wounds

Part II. Evaluation of agents which have shown cytologic activity

Project Description:

Part I. Significance of tumor cells in operative wounds

Objectives:

The subject of cancer cells seeding of operative wounds has captured the interest of surgeons since the turn of the century. Attention was re-directed to this subject in 1955 when Engell reported a high incidence of tumor cells in the peripheral blood of patients undergoing cancer surgery.

Since 1957 the Surgery Branch has been involved in evaluating the incidence, significance and prevention of tumor cell seeding of wounds. Previous reports, however, have failed to note any relation between the incidence of

tumor cells in wound washings and the future development of local wound recurrences. These studies called for the examination of saline washings of the operative wounds just prior to closure. However, on occasion, drainage material from wounds collected after surgery were similarly surveyed and malignant cells found as late as 3-5 days postoperatively. This suggested that wound drainage examination would provide a more sensitive test for the thesis that patients with tumor contamination of wounds run a greater risk of local recurrence.

Methods Employed:

One hundred and sixty-seven patients undergoing radical cancer surgery from 1960 to 1964 have had simultaneous wound washing and wound drainage studies done, with particular reference to the presence of malignant cells. Patients have been followed from 1 to 5 years. Wound washings were collected at surgery prior to closure. Wound drainage specimens were collected by means of a suction catheter leading to a trap bottle immersed in ice. The bottle contained 40 mg. of heparin, 20,000 units of streptokinase and 5,000 units of streptodornase to improve the filterability of the fluid. Samples were collected and bottles changed every three hours for the first 12 post-operative hours, then every eight hours for the next 24 hours, every 12 hours for the next 24 hours and finally once daily until the drainage catheter was removed.

The specimens collected from the first 108 patients were processed according to the technique of Malmgren et.al., using streptolysin O and millipore filters. The remaining cases were processed by one step filtration method of West and associates utilizing the Synpor 14 micron filter. The recovery rates of both techniques were appraised by seeding known negative drainages with 1,000 tumor cells as determined by hemocytometer count and found to be comparable. The second method was adopted solely for its extreme simplicity. The filters, after staining and mounting, were screened by technologists. Positive and suspicious cells were marked and confirmed by a cytologist. Particular attention was given to mesothelial cells and fibroblasts which might be sufficiently atypical to cause confusion with neoplastic cells.

Major Findings:

The accumulated data is only in the early stages of compilation. However, several preliminary observations have been made. First, the incidence of tumor cells in wound washings (10%) is far lower now than in previous series (approximately 26%) reflecting either better operative technique or greater selectivity in classifying cells as malignant. Second, although tumor cells may be found as late as 159 hours after surgery, even when the washings at the time of surgery were negative, there appears to be no correlation between positive drainages and the future course of disease. Wound recurrence rates were quite similar in the two groups and many with evidence of wound seeding have been followed without sign of recurrent disease.

LOCAL RECURRENCE RATES

	Number of pts.	Local recurrence
All specimens negative	116	27
Wound drainages positive	16	7
Wound washings positive	16	6

Significance to Bio-medical Research and Program of the Institute:

The meaning of the disseminated cancer cell in cancer patients undergoing surgery remains elusive. Surgeons continue to observe many precautions with respect to the prevention of contamination, butthere is little or no experimental evidence to support the need for these practices. It would seem that as yet undefined mechanisms of host resistance play a far greater role. It is also important to realize that the methods described herein and used by others are relatively insensitive, since only 50% of patients with unresectable lesions had positive operative wound washings.

Proposed Course of Project:

Complete data analysis for the entire wound washing program of all patients submitted to cancer surgery for the past five years.

Project Description:

A continued evaluation of agents which have shown cytologic activity either in animal or human test systems with an attempt to effectively control the growth of tumor in wounds contaminated with tumor cells.

Part II. Evaluation of agents which have shown cytologic activity

Methods Employed:

Drugs are selected which the literature, or our personal clinical or laboratory experience suggest may have significant anti-bacterial or tumoricidal activity. Toxicity studies, by dilutional techniques, with the agent tested both intraperitoneally and topically determine the concentration and volume of fluid to be tested. Animals' open wounds are then "seeded" with tumor cell inoculums of standard cell count and volume. The test agent is applied with variants of pressure at variable time intervals after wound contamination. The wounds are closed and the animals followed for tumor growth and compared with control animals not irrigated and control animals irrigated by saline (mechanical effect).

Major Findings:

We now have tested over 50 agents and variable concentrations of these agents. Proflavine hemisulfate, an acridine dye, has consistently shown the best tumoricidal activity. The somewhat toxic concentration of 50% alcohol and a

2% Dakins solution have shown decreased tumor growth to a 20% level as compared to 40-60% incidence of wound regrowth of tumor with most other agents tested. Proflavine hemisulfate has little toxic effect in the open wound.

The mode of application is also important in the interpreatation of results. The greater the presssure used in applying the irrigant, the higher percentage of tumor takes. For example, simple syringe irrigation is much more effective than is the spray technique whereby the irrigant strikes the seeded wound with 5 mm. of pressure or greater.

Significance to Bio-medical Research and Program of the Institute:

Very possibly the many reports in the literature showing varied effects of different agents on the prevention of animal wound seeding in some instances, may be based on the mode of application of cytoxic agents. If implants of tumor can be driven into the tissues by certain techniques of wound washing, then this is of great significance to the clinician in the manner in which he handles his operative wounds. The continued testing of numerous agents under this standard test system may eventually prove to be valuable in the clinic.

Proposed Course of Project:

Proflavine hemisulfate has been shown to be chemically suitable to clinical trial. Three hundred forty-eight patients have now been entered into a clinical program of prophylactic wound washing following cancer surgery. The results await the time interval which is necessitated by clinical experience in determining any signficance decrease in tumor cell contamination of wounds.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Fisher, J.C., Ketcham, A.S., Hume, R. and Malmgren, R.A.: The significance of cancer cells in operative wounds. Am. J. Surg. 114: 514-519, Oct. 1967.

Serial No. NCI-3805

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: The Experimental Studies of Mechanisms of Metastases

Previous Serial Number: Same

Principal Investigator: Dr. Alfred S. Ketcham

Other Investigators: Hilda Wexler, Drs. John R. Marshall, William G. Hammond, Jack C. Fisher, Robert C. Hoye, Paul B. Chretien, James J. Ryan, John D. Harrah, Frank C. Sparks and Harold R. Gertner.

Cooperating Units: Pathologic Anatomy Branch, NCI

Man Years: (July 1, 1967 through June 30, 1968)

Total:	
Professional:	1 1/2
Other:	1
	1/2

Project Description:

Part I. The effect of prolonged anticoagulation on spontaneous tumor metastases.

Part II. Growth of tumor in isolated perfused organs

Part III. Differences in immunogenicity between primary tumor and their metastases.

Part IV. The experimental study of metastases

Project Description:

Part I. The effect of prolonged anticoagulation on spontaneous tumor metastases.

Objectives:

1. To evaluate the effect of long-term anticoagulation on the size of a primary tumor and its spontaneous metastases in an isologous host system.

2. To observe morphology and fibrin content of a tumor growing in an anticoagulated animal. The coagulation mechanism is believed to play a role in the initial adherence of embolic tumor cells and subsequent development of distant metastases. Transient anticoagulation decreases the number of pulmonary metastases in animals given tumor cells intravenously. This study evaluates the effect of long-term anticoagulation on the size of a primary tumor and its spontaneous metastases in an isologous system.

Methods Employed:

Two host-tumor systems were studied. The methylcholanthrene sarcoma T241 of Lewis in C57 black 6N mice and the spontaneous mammary adenocarcinoma in C3H/HeN mice. Following tumor innoculation one-half of the mice in each system were begun on oral sodium warfarin at a constant pre-determined dosage. At five intervals equal numbers of control and anticoagulated mice were sacrificed after prothrombin time determinations. By India ink differential staining of tumor and lung tissue, metastases were detected macroscopically.

Major Findings:

Prothrombin times of the mice were maintained continuously in the range of 2 to 3 times normal and the mice free of any hemorrhagic complications. In each of the five intervals of comparison in both host-tumor systems at least three times as many control as anticoagulated mice had metastases. For the C57 mice a cumulative total of 73% of controls and 15% of anticoagulated mice had metastases and for the C3H system, 62% of controls and 15% of these anticoagulated.

Significance to Bio-medical Research and Program of the Institute:

The results support the earlier work done with an early generation methylcholanthrene induced fibrosarcoma. In all three systems long-term anticoagulation has significantly reduced the incidence of spontaneous metastases and significantly increased the number of individuals free of metastatic disease. The more recent studies show this effect of anticoagulation will persist when the level of anticoagulation is maintained constantly in a safe range free of any hemorrhagic complications. This further attests to the importance of the coagulation mechanism in the intravascular metastatic spread of tumors.

Proposed Course of Project:

The use of anticoagulation as an adjunct to standard therapy with surgery or irradiation is being evaluated.

Project Description:

Part II. Growth of tumor in isolated perfused organs

Objectives:

1. Culture of selected animal and human tumors in vitro as a means for testing chemotherapeutic agents.

2. Culture of animal and human tumors in vitro and study of factors believed relative to metastases formation.

Methods Employed:

The technique used is that developed by Dr. Judah Folkman of the Harvard Fifth Surgical Service, Boston City Hospital, Boston, Massachusetts. The thyroid gland or an isolated segment of small bowel is removed from a small dog and the artery is cannulated. These organs are then perfused with an acellular media. They are placed in a small glass organ chamber and the temperature is maintained at 37° by means of a constant temperature incubator. Use of an acellular media containing balanced electrolytes, fetal calf serum and essential amino acids along with antibiotics and vitamins will keep the organ alive for several days and allow the growth of heterologous tumor without cellular rejection. We hope to be able to grow certain human tumors such as choriocarcinoma of the uterus and carcinoma of the breast. We would then be able to test its response to various hormones and to chemotherapeutic agents. In addition, thru the use of both animal and human tumors, we hope to study the cell shedding rate, cohesiveness and other factors believed relative to the formation of metastases. In this manner it might be possible to determine which tumor is likely to metastases early in the course of the cancer spectrum.

Major Findings:

At the present time we are learning the technique and have had survival of some of the implanted tumor for periods of up to 10 days, but no progressive growth.

Significance to Bio-medical Research and Program of the Institute:

This technique has far reaching possibilities. If it would be possible to determine the type and nature of a malignancy early in the course of the patient's disease, rational therapy could be planned. The technique is also applicable to many immunological studies.

Proposed Course of Project:

It is hoped that we will be able to grow a series of 1 or 2 types of tumors and characterize their behavior in vitro.

Project Description:

Part III. Differences in immunogenicity between primary tumors and their metastases.

Objectives:

To test the hypothesis that a mechanism of immunoselection is operative among those cells shed from the primary tumor into the blood stream and that the less immunogenic cell or cells are those that develop as metastases.

Methods Employed:

Metastases and primary tumor from the same animal are separately transplanted into groups of animals, and the tumor-bearing legs amputated when the tumors are 1.0 cm. in diameter or greater. Seven to ten days later equal doses of each tumor line are given to control mice and to mice immunized with either the primary or metastatic tumor line. The development of tumors at the site of challenge injection is noted. Comparison between control mice and those previously bearing one of the tumor lines is made statistically. Accelerated growth of tumor is indicative of enhancement and decreased growth demonstrates immunity.

Major Findings:

In the four tumor-host systems thus far studied, differences in immunogenicity have been shown between primary and metastatic tumor lines. Although there are some exceptions related to varying challenge doses, in general the primary tumor appears to be more immunogenic.

Significance to Bio-medical Research and Program of the Institute:

As progress is made towards effective immunotherapy for human cancer, it becomes more clear that the major target initially will be metastatic disease. It, therefore, is of importance to know whether immunologic measures directed against primary tumor antigens will be effective against metastatic lesions.

Proposed Course of Project:

Further studies will be done with other tumor-host systems, particularly with different methods for evaluating tumor-specific immunogenicity. Preliminary studies have suggested that cutaneous delayed hypersensitivity reactions in the guinea pig will be applicable, as will an in vitro method for quantitation of tumor-specific humoral antibody now undergoing validation in this regard.

Project Description:

Part IV. The experimental studies of metastases

Objectives:

The biological behavior through successive transplant generations of transplantable tumor derived originally from primary chemically induced and

spontaneous sources in mice has been studied to document the changes in the behavior of three induced and two spontaneous tumors in four different strains of mice that occurred over several transplant tumor generations. Considered in this documentation were: the primary tumor growth rates, survival time of mice with intact primary tumors the incidence of pulmonary and extropulmonary metastases in both intact and amputated tumor mice and the changes that occurred in these parameters with preceding transplant tumor generations.

Methods Employed:

Three chemically induced and two spontaneous tumors were studied C57/BL/5N and Balb/cAnN mice were injected with 1- 1/2 cc. of ethiodol. Mice injected with both carcinogens developed fibrosarcoma. The spontaneous tumors studied were mammary adenocarcinoma arising in exbreeder C3H/HeN mice and a fibrosarcoma that arose in a single DBA/2N mouse. Tumors were transplanted by the injection of cytosieve suspension into the right hind leg muscles of mice when the tumors in each group reached 1.5 - 2.0 cm. in diameter, approximately 1/2 of the mice had their right tumor bearing leg amputated by a cautery clamp method, in the remaining mice, the tumors were left intact. After tumor amputation all animals were housed individually. When the last intact tumor mouse of a group died, the remaining amputated mice in the paired group were killed. A complete autopsy was performed on all animals. Pulmonary metastases were identified using the India ink method. Metastases in other body sites were recognized grossly. Complete data were collected in the 1st, 3rd, 5th, 7th, 11th and 23rd transplant generations of all tumors. The 3 methylcholanthrene induced tumor in the C57BL/6N mice and the ethiodol induced tumor in Balb/cAnN mice were further extended in the 26th and 27th transplant generation. In addition, data were collected on 30 C57 and 40 Balb/cAnN mice with original 3 methylcholanthrene induced fibrosarcoma and 17 C3H/HeN mice with spontaneous mammary adenocarcinoma.

Major Findings:

Studies done on the survival rates of tumor-bearing mice in all tumor systems shows that there is a variation in the growth rate of the primary tumor and in pulmonary and extrapulmonary metastases of a period of several transplant tumor generations. From its inception each tumor has its own malignant pattern on the basis of these two parameters. In general, induced tumors are more malignant than spontaneous tumors. After a number of transplant tumor generations the tumor behavior is more predictable. The time it takes to reach this pattern varies with the individual tumor. The virulence of a tumor can be determined in both tumor-bearing and amputated tumor mice. The present investigation revealed that surgery reduced the numbers of mice with metastases and the number of metastases in each mouse. Contrary to results of previous reports we found that original carcinogen-induced tumors and spontaneously arising tumors do metastasize when the

animals survive long enough for cells to shed from the original tumor.

Significance to Bio-medical Research and Program of the Institute:

Experimental metastases is a long-term study of the branch which is attempting to clarify some of the bizarre behavior patterns seen in tumor development in humans. Certain associations between the growth and metastasizability of malignant tumors in laboratory animals and of malignant disease in humans have been noted. There are many clinical concepts which can be duplicated in the laboratory animal through which a more definitive understanding of the bizarre behavior of cancer has been obtained. It remains apparent, however, that malignant disease, whether in humans or laboratory animals, must be individualized. This makes the interpretation of data and the correlation of results difficult.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Orme, S.K. and Ketcham, A.S.: Effect of prolonged anticoagulation on spontaneous metastases. Surg. Forum 18: 84-85, 1967.

Wexler, H., Orme, S.K. and Ketcham, A.S.: The biological behavior through successive transplant generations of transplantable tumors derived originally from primary chemically-induced and spontaneous sources in mice. J.N.C.I. 40: 513-523, March 1968.

Serial No. NCI-3806

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Surgical Physiology and Metabolic Changes Associated with Extensive Cancer Surgery

Previous Serial Number: Same

Principal Investigator: Dr. Robert C. Hoye

Other Investigators: Drs. David F. Paulson, Kenneth B. Gray, Alfred S. Ketcham and Frederick R. Eilber

Cooperating Units: Department of Anesthesiology and Blood Bank, Clinical Center, NIH

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1 1/2
Professional:	1
Other:	1/2

Project Description:

Part I. The study of fluid volume alterations occurring in cancer patients before and after extensive surgery.

Part II. The study of albumin loss and sequestration in man before and after extensive surgery.

Part III. The study of composition and transport of lymph in man before, during and after periods of surgical stress.

Part IV. The effect of hemorrhage and trauma on fluid partition and protein movement in the experimental animal.

Part V. The quantitation of plasma albumin sequestration by the gastrointestinal tract following standardized abdominal trauma in patients and animals.

Part VI. Surgical morbidity following major pelvic surgery as related to blood transfusion.

Project Description:

Part I. The study of fluid volume alterations occurring in cancer patients before and after extensive surgery.

Objectives:

To investigate changes in total red cell volume, plasma volume and extracellular fluid volume, occurring in patients during extensive surgical procedures. Changes in these parameters are to be correlated with changes in the patient's clinical course as manifestated by the usual clinical signs of pulse, blood pressure, urine output, central venous pressure measurement and peripheral hematocrit.

An additional objective of this portion of the project is to provide a service for other investigators of the Clinical Center for the accurate measurement of total red cell volume and plasma volume on their patients for either diagnostic or study purposes.

Methods Employed:

The methods employed have been developed in this laboratory and are capable of simultaneously measuring the total red cell volume with chromium-51, plasma volume with iodine-125 and extracellular fluid volume with sulfur-35 sodium sulfate. With this method, the error in space measurements in patients is less than 3%. Volume measurements are made in patients 24 hours preoperatively and again immediately postoperatively. During surgery, monitoring of the central venous pressure is done with an inlying subclavian vein catheter and blood loss is measured in an electronic blood loss meter.

Major Findings:

This continuing study has demonstrated that marked deficits frequently are present in patients prior to elective surgery and that further deficits occur during the course of operation. Some patients came to surgery with deficits in the total red cell volume as high as 38% of their calculated normal value. In the operating room the volume of blood lost was frequently underestimated. When accurate measurements were carried out between 15 and 42% of the total operative loss was found on the drapes and gowns. In the postoperative period loss of red blood cells continued and averaged 22% of the immediate postoperative total red cell volume.

The question of partitioning of the total red cell volume following extensive surgery into rapidly circulating, slowly circulating and non-circulating components was investigated. There was found to be no evidence for partitioning of the total red cell volume (TRCV) into separately circulating components even after the most extensive surgery. We did find in analyzing this data, that a disproportionate deficit of the plasma volume occurred

after major surgery which appeared to be proportional to the magnitude of the surgical trauma. In patients undergoing pelvic exenteration, this plasma volume deficit amounted to 25% of the preoperative value. This disproportionate plasma volume deficit was not apparent in patients undergoing extensive surgery in the head and neck area suggesting that the responsible agent is intra-abdominal manipulation.

In 15 patients undergoing extensive abdominal surgery, the total circulating protein was found to decrease during the period of operative trauma in all of the patients for an average decrease of 33% of the starting value. In 8 consecutive patients having pelvic exenteration, a significant deficit occurred in the effective extracellular fluid volume as compared to values obtained 24 hours preoperatively. This fell in each of the patients and averaged 15% of the starting value.

These latter two observations suggest that the loss of plasma protein and extracellular fluid are responsible for the disproportionate plasma volume deficit which occurs.

These marked deficits have been manifested clinically by a cool vasoconstricted patient with a low central venous pressure, slightly elevated pulse, normal blood pressure and low urinary output. Serial microhematocrit determinations during the course of an extensive procedure and immediately following have not been helpful as a guide for replacement therapy.

Significance to Bio-medical Research and Program of the Institute:

Metabolic changes following extensive cancer surgery have not previously been thoroughly investigated. To date these quantitative studies in patients undergoing extensive cancer surgery have indicated that a multiplicity of deficits may occur. It is anticipated that with increased knowledge in this field both the morbidity and mortality which currently occurs following some operations for the removal of cancer will be appreciably reduced.

Since the start of these studies the management of the patient undergoing extensive cancer surgery has changed drastically. Any deficits which exist preoperatively are replaced prior to the time of operation. In the operating room additional electrolyte containing fluids are administered along with additional albumin solution so as to maintain the effective extracellular fluid volume and prevent the disproportionate plasma volume deficit mentioned above. Blood loss is more accurately monitored in the operating room and in the immediate postoperative period. Quantitative replacement is accomplished before the onset of clinical signs indicating decompensation.

Proposed Course of Project:

Future studies will continue to investigate these parameters in selected patients while manipulating the type of replacement fluids given during the period of surgical stress.

Project Description:

Part II. The study of albumin loss and sequestration in man before and after extensive surgery.

Objectives:

To determine the response of the total body albumin pool (total exchangeable albumin) and its component parts to major surgery. Previous studies have indicated that a contraction of the plasma volume and extracellular fluid volume occur with major surgery. It has also been demonstrated in these patients that there is an average decrease of 33% in the total circulating protein. The existence of such marked protein deficits has not previously been described and this study is designed to investigate in a quantitative fashion this protein deficit.

Methods Employed:

Patients selected for this study will have total body albumin pool measurements performed by injecting radioactive human serum albumin, 7 to 10 days preceding surgery. By following the daily plasma level of the albumin tag and urinary excretion, the amount of albumin degraded daily can be obtained. The point of equilibrium distribution of the albumin between intravascular and extravascular compartments can also be determined. Postoperatively, a second albumin tag will be administered and the total body albumin pool again determined in a similar fashion. The patients selected for this study are in a steady state preoperatively and again postoperatively at the time the albumin measurement is carried out.

Major Findings:

To date 16 patients have been studied. Five of these patients served as control patients while 11 underwent pre- and postoperative determinations of the total body albumin pool. In six patients having a uniform type of surgical procedure, that is, regional node dissection, the findings were similar. These six patients had normal total exchangeable albumin pools preoperatively. Following surgery there was a 36% decrease (range 26 to 48%) in the net total exchangeable albumin pool which represented an albumin deficit postoperatively of 96 grams. This deficit occurred despite adequate transfusions at the time of surgery to replace operative loss. When the total body albumin pool was proportioned into the respective intravascular and extravascular compartments, there was found to be a 27% decrease in the intravascular compartment as compared to a 43% decrease in the extravascular compartment. This data suggests that the body attempts

to maintain the intravascular compartment at the expense of the extravascular albumin space. In two patients studied, the administration of albumin solutions during or immediately following surgery made these deficits less marked. The reason for these deficits is not entirely clear. The loss may be occurring externally at the time of surgery, yet in two patients when the total loss of albumin on sponges and postoperative drainage was measured, it accounted for less than 50% of the net total exchangeable albumin deficit present postoperatively.

Significance to Bio-medical Research and Program of the Institute:

The immediate significance of this work is improvement of the pre- and postoperative management of patients with major surgical disease. This study suggests the reason for the disproportionate plasma volume deficits previously reported and in addition indicates that an albumin deficit occurs in both the intravascular and extravascular pools postoperatively. For several years we have found it necessary to administer large quantities of albumin to patients following extensive cancer surgery and this study is one of several we are conducting which puts this empirical type of treatment on a firm basis.

Proposed Course of Project:

Current studies employing the administration of iodine-131, serum albumin and whole body counting are being done in an attempt to identify this postoperative deficit in total exchangeable albumin to be secondary to an internal loss or sequestration of albumin in a sight not available for exchange with the total body pool. A series of patients having surgery of a similar magnitude will be studied in whom albumin is administered during surgery. Similar postoperative measurements will be carried out to see if the total exchangeable albumin pool can be maintained near normal with this treatment regime.

Project Description:

Part III. The study of composition and transport of lymph in man before, during and after periods of surgical stress.

Objectives:

Previous studies in this laboratory have indicated that a marked deficiency in the total circulating protein occurs during the time of extensive surgery. Since the lymphatic system is the main avenue by which protein is returned from the extracellular fluid to the intravascular compartment, it was felt that a study of lymphatic compartment, it was felt that a study of lymphatic composition and transport might demonstrate the reason for the disproportionate plasma volume deficit, and total circulating protein deficit which has been observed. Other investigators have indicated

in dogs that during a period of hemorrhage there is an increase in the flow and the protein content of lymph returning to the intra-vascular compartment. It is the objective of this study to investigate the mechanism in humans, during the period of operative stress and hemorrhage by which extravascular protein is returned to the intravascular compartment.

Methods Employed:

Selected patients upon whom it is planned to perform extensive cancer surgery will undergo cannulation of the left thoracic duct four days prior to surgery. In the ensuing two days after an adequate lymph flow has been established equilibration studies with iodine-125 and sulfur-35 are carried out. During the period of operative stress, the volume flow and protein content of the lymph are monitored and later correlated with plasma volume changes. Through the equilibration studies which are performed pre-operatively, it is anticipated that we will be able to determine the relative size of the lymphatic compartment and its albumin content.

Major Findings:

Studies to date have indicated that during extensive surgery the volume of lymph flow per unit time as well as the concentration of albumin in the lymph in grams per 100 ml. decreased during the course of surgery. During the course of surgery the amount of albumin returned to the vascular compartment per unit of time was 25% of that which has been returned during preoperative baseline studies. This suggests one reason for the deficit in total circulating protein and disproportionate plasma volume loss which have previously been described.

Significance to Bio-medical Research and Program of the Institute:

These studies on the composition and flow of lymph in man during period of operative stress indicate that the response of the lymphatic system to hemorrhage and trauma in man is somewhat different from that previously recorded in the dog. Continued investigation of these parameters should provide guidelines which will permit more adequate homeostatic maintenance of patients during extensive surgery.

Proposed Course of Project:

During the past year this study has been seriously curtailed by the need to cut back in overtime work. Our laboratory is now able to perform the necessary chemical and electrophoretic protein analysis formerly done in clinical chemistry. Continued investigation of patients along the lines outlined above will continue to more firmly establish the validity of the preliminary findings mentioned. It is anticipated that measurements of the patient's total exchangeable albumin before and after surgery will provide additional information in these investigations.

Project Description:

Part IV. The effect of hemorrhage and trauma on fluid partition and protein movement in the experimental animal.

Objectives:

Advances in the knowledge in the pre- and postoperative care of patients undergoing extensive surgery is continually hampered by the lack of good experimental models. The dog has been used for many years in the study of post-traumatic physiology. Exstrapolation of results obtained in the dog to the human is however not without danger as many of the basic hemodynamic responses in the dog do not occur at the same rate as they do in the human. The objective of this study is to establish in the baboon an experimental model of extensive operative trauma which closely approximates the clinical situation. By so doing the effect of various treatment regimes on altering fluid compartments and protein movement can then be determined.

Methods Employed:

Adult male baboons will be employed in the study. The standard operative trauma will consist of a pelvic and retroperitoneal node dissection. Following the surgery the animal will be immobilized in a support for one week to permit the performance of postoperative studies. The size of various fluid compartments will be measured before and at varying time intervals after surgery. The affect of administering various fluid regimens and protein solutions on fluid partition and protein movement during and following surgery than can be determined. This investigation is only currently being started and no major results have yet been obtained.

Significance to Bio-medical Research and Program of the Institute:

It is anticipated that this experimental model will provide in a short period of time answers to pertinent questions such as the following:

1. Is postoperative weight gain necessary following extensive surgery with adequate fluid replacement?

2. Is albumin administration during the course of surgery necessary or desirable?

3. What is the effect of a large volume of salt solution during surgery on
   a) the extracellular fluid volume?
   b) the intravascular albumin content?

Proposed Course of Project:

When fully underway it is anticipated that this project will involve the

major portion of the investigative interest of this laboratory.

Project Description:

Part V. The quantitation of plasma albumin sequestration by the gastrointestinal tract following standardized abdominal trauma in patients and animals

Objectives:

This study has two major objectives; one is to identify the quantity of albumin which has sequestered into the bowel wall and bowel lumen following a standard operative procedure. Secondly, we wish to determine the effect of time upon the sequestration and reutilization of this sequestered albumin.

Methods Employed:

Chromium-51 labelled human serum albumin has been demonstrated to be an accurate indicator of gastrointestinal albumin loss. The chromium-51 label is neither secreted nor absorbed by the GI tract to any significant extent so that a measure of the chromium-51 activity in the stool can be related to the serum equivalents of albumin when one knows the specific activity of 1 cc. of serum. In like manner chromium-51 activity determined to be in the wall of the bowel and can be identified as serum equivalents of albumin. Patients selected for this study are those undergoing a similar type of extensive intra-abdominal operative procedure, that is, radical hysterectomy. The labelled albumin solution is being given 8 days preoperatively and the daily loss in the stool is followed. Postoperatively the stool collections continue and the intraluminal loss of albumin as a result of the surgical procedure can then be determined. By concomitantly administering iodine-125 tagged albumin the total exchangeable albumin pool preoperatively can be determined and the intraluminal loss of albumin then represented as serum equivalents or a certain percentage of the daily albumin degradation rate. At the same time two concomitant groups of patients are being studied: 1) an operable control group having only minor abdominal surgery and 2) an "anoperated control group" who are undergoing surgery in an area other than the abdomen.

To determine the loss of albumin into the wall of the gastrointestinal tract with extensive abdominal surgery it is necessary to utilize animals. Male Rhesus monkeys of similar weight and age have been selected for study. Prior to surgery all animals are injected with iron 59 labelled red blood cells and chromium-51 labelled serum albumin. Following equilibration of the labelled albumin throughout the intravascular and extravascular compartments the animal is subjected to a standardized amount of intra-abdominal trauma. This trauma is intended to simulate that which occurs with extensive intra-abdominal operative procedures. Six hours following surgery, the animal is again operated upon and the entire gastrointestinal tract removed. The bowel wall and luminal contents are then homogenized

and the chromium-51 activity contained in an aliquot of each homogenate is then determined. The iron 59 activity is simultaneously counted with the chromium-51 so as to identify the amount of sequestered albumin which is secondary to retained whole blood. Three groups of animals are being studied. The experimental group: (1) undergoes laparotomy with standardized intestinal trauma. The animals in group (2) are subjected to laparotomy only without intestinal trauma and in group (3) no abdominal surgery is performed.

Major Findings:

Work on this project is still in the developmental phase. One patient has been studied who underwent a laryngopharyngectomy with radical neck dissection. This patient was found to lose 41 cc. serum equivalents of albumin into the gastrointestinal tract during the 5 days preceding surgery and 809 cc. serum equivalents into the gastrointestinal tract on the 5 days following surgery. This high postoperative loss was unexpected in this patient and may have been due to a leakage of blood at the pharyngeal suture line. Preliminary studies performed in the animals have indicated that as much as 40% of the plasma volume is sequestered into the bowel wall and lumen at the time of surgery.

Significance to Bio-medical Research and Program of the Institute:

Previous studies in this laboratory have identified marked deficits in the circulating albumin occurring with major surgical procedures. A large proportion of this loss seems to be due to an internal sequestration. If the site of this sequestration can be identified and quantitated the maintenance of normal homeostasis during surgery will be facilitated. This project is directed at the recognition of the site of loss so that adequate prevention and rational treatment might be instituted in the clinical situation.

Proposed Course of Project:

As patients become available they will be included in the study. The animal study will continue until at least 20 animals have been included. If the results continue to be as significant as in the preliminary trials, additional studies investigating the time of mobilization of this sequestered albumin will be undertaken.

Project Description:

Part VI. Surgical morbidity following major pelvic surgery as related to blood transfusion.

Objectives:

This study was designed to investigate the morbidity related to bleeding in

all patients having extensive pelvic surgery over the preceding 18 months. The intent was to see if bleeding difficulties could be related to the amount or condition of the blood the patient received during the time of surgery.

The blood bank has need for many platelet concentrates and we wish to see if the excessive bleeding occurring during the course of an operation could be related to administration of whole blood which had previously been centrifuged and had the platelets removed.

Methods Employed:

This was a retrospective analysis of the charts of all patients undergoing major pelvic surgery during the preceding 18 months. Factors evaluated include the presence or absence of prior irradiation treatment, whether there seemed to be excessive bleeding during, or immediately following surgery; the mean and median age of the first 10 units of blood given the patient during the surgery, the number of these 10 units which had previously been centrifuged and platelets removed; and the total units of blood received in the operating room in the first 12 hours postoperatively.

Major Findings:

The charts of 17 patients who had undergone extensive pelvic surgery were reviewed. These revealed the following: Five patients had had difficulty with severe bleeding during and immediately following the surgery; six patients had a moderate amount of abnormal bleeding during and immediately following the surgery; and six patients displayed no abnormal bleeding either during the time of surgery or immediately following. All five of the patients in the severe bleeding category were radiation failure patients while only 1/2 of the patients in each of the other two groups were radiation failure patients.

Of the factors evaluated, the most prominent appeared to be the age of the blood transfused at the time of surgery. In those patients who demonstrated no evidence of abnormal bleeding at the time of surgery, the median age of the first 10 units of blood received was between 2 and 8 days. In the 13 patients demonstrating a moderate to severe bleeding abnormality at the time of surgery, the median age of the first 10 units of blood received in the operating room was 4 to 20 days. In 7 of these 13 patients the median age was between 15 and 20 days.

The effect of prior centrifugation of the blood with removal of platelets was difficult to evaluate in this retrospective analysis. Four of the 13 patients with abnormal bleeding and one of the six patients with no abnormal bleeding had received over 5 units of centrifuged blood in the operating room. Of possible significance is the fact that the one patient in the group with no abnormal bleeding was transfused with 9 units of

centrifuged blood in the operating room having a median age of 3 days, While the median age of the centrifuged blood given the other four patients varied from 4 to 18 days. This suggests that centrifugation with removal of platelets may not be a factor contributing to abnormal bleeding if the blood is less than 3 days old when administered.

Significance to Bio-medical Research and Program of the Institue:

Occasionally bleeding diatheses occur in surgical patients which can not be explained. It is important to know if the blood transfused at the time of surgery is the causative agent. Other programs in the institute require a large number of platelet concentrates. If blood from which the platelets have been removed can be used at the time of surgery then further use will be made of the blood transfusion products currently available.

Proposed Course of Project:

As a result of this preliminary investigation the Clinical Center Blood Bank has embarked on a project to evaluate the clotting factors and fragility of the red blood cells in bank blood which has been subjected to centrifugation and removal of platelets. Future patients undergoing extensive pelvic surgery will be transfused with blood which is less than one week old whenever possible. During the course of surgery, one unit of fresh blood will be administered for every five units of bank blood transfused.

Depending upon the results of the in vitro study now being performed it is anticipated that a prospective analysis will be carried out on two groups of patients undergoing major surgery. One group will receive blood less than 3 days old from which platelets have been removed and the other group will receive blood with the platelets intact.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Hoye, R.C. : Simultaneous measurement of blood and extracellular fluid volume in the surgical patient. J. of Lab. & Clin. Med. 69: 683-688, Apr. 1967.

Hoye, R.C., and Ketcham, A.S.: Shifts in body fluids during radical surgery. Cancer 20: 1827-1831, 1967.

ARTICLE IN PRESS:

Klein, L.A., Roth, J., and Gordon, P.: Antidiuretic hormone metabolism in man. (J. Urol.)

Serial No. NCI-3808

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Study of Host Factors Associated with Implantation and Growth of Cancer

Previous Serial Number: Same

Principal Investigators: Dr. Paul Chretien, Dr. William G. Hammond, Dr. Donald L. Morton

Other Investigators: Dr. H.B. Andervont, Dr. Costan W. Berard, Dr. Winthrop Churchill, Dr. T.B. Dunn, Dr. John Fahey, Dr. Phillip Grimley, Dr. Walter Heston, Dr. E.C. Holmes, Roxane Hume, Dr. Alfred S. Ketcham, Dr. Richard Malmgren, Dr. Alan Rabson, Dr. Paul Schmidt, Dr. George Vlahakis, Dr. John D. Harrah

Cooperating Units: Pathologic Anatomy Branch, Immunology Branch, Biology Branch and Laboratory of Biology, NCI; Blood Bank, Clinical Center

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1½
Professional:	1
Other:	½

Project Description:

Part I. Evaluation of tumor-specific immunity in new isologous tumor-host systems

Part II. Immunology of chemically-induced tumors in inbred rodents

Part III. Feasibility studies of immunotherapy for solid tumors

Part IV. Immunobiology of the lymphoid tissues in inbred rodents with chemically-induced tumors

Part V. Immunologic relationships among murine mammary tumor agents

Part VI. Acquired immunological tolerance in carcinogenesis by the mammary tumor virus

Part VII. In vitro stimulation of autochthonous lymphocytes by the host tumor

Part VIII. Immunologic studies with human neoplasms

Project Description:

Part I. Evaluation of tumor-specific immunity in new isologous tumor-host systems

Objectives:

To obtain and immunologically evaluate various experimental tumor-host systems more closely akin to human neoplasms than some of the previously studied lesions.

Methods Employed:

Efforts have been made to induce tumors of the desired sort in inbred rats with dimethylbenzanthracene, cycasin, and 2,7-acetylaminofluorene given orally. A transplantable rat osteogenic sarcoma has been obtained, and will be evaluated by the methods mentioned below. Currently, rats are being given 3,2'-dimethyl-4-aminobiphenyl, a carcinogen reported to give a high yield of large bowel neoplasia. A transplantable bile-duct adenocarcinoma of strain 2 guinea-pigs has been studied by the tumor-amputation-challenge method of immunogenicity testing and by the method of intradermal injection of tumor cells following immunization by tumor amputation. In this latter method, development of delayed cutaneous hypersensitivity reactions is indicative of immunity, and in both methods tumor growth at the challenge site is compared between experimental rat control groups to evaluate immune response.

Major Findings:

The yield of rat tumors has been most disappointing. However, the guinea-pig bile-duct adenocarcinoma has been shown to be immunogenic by both of the methods used for testing. This tumor metastases via the lymphatics, a property uncommon in experimental rodent tumors but which will make this a most useful tumor-host system for many types of experimental tumor work.

Significance to Bio-medical Research and Program of the Institute:

The acquisition of two new transplantable tumors useable in large rodents provides new tools for studies of tumor immunology in animals large enough to allow a wider variety of procedures than is feasible in the mouse. Also, these lesions more closely resemble human disease than do some of the mouse tumors, and it is believed that principles derived from the study of experimental tumors will be applicable to human disease in direct proportion to their similarity to the human lesion.

Proposed Course of Project:

Kinetics of the immune response will be studied in both the new systems. The search for more approximate tumor-host systems is continuing.

Project Description:

Part II. Immunology of chemically-induced tumors in inbred rodents

Objectives:

To delineate more clearly the mechanism whereby tumors produce tumor-specific transplantation immunity and to ascertain the mechanism whereby such immunity is made effective.

Methods Employed:

The basic method utilized is the tumor-amputation-challenge technique, in which mice receive an injection of tumor suspension in a hind leg, undergo amputation of the tumor-bearing leg after tumor has developed, and are then challenged later in the opposite leg with known doses of a cell suspension prepared from the tumor being evaluated. Controls included normal mice and mice having had amputation of a leg bearing a different syngeneic tumor. The end-point is the development of palpable tumor at the site of challenge injection.

The role of hemolytic complement in the development and manifestation of syngeneic tumor immunity was studied by the above method in two systems. The first was the B10-D2 "old" and B10-D2 "new" strains of mice. These are generally believed to be syngeneic but the B10-D2 "old" line is deficient in whole complement activity on the basis of a specific C'5 component deficiency. Tumors were induced in both strains with methylcholanthrene and tested in both strains. The second was the system AL/N - A x ALF_1 - C x ALF_1, in which the first two strains of mice are similarly complement deficient but the third has normal complement activity. Tumors induced in the common parental strain, AL/N, are readily transplantable into both F_1 strains without interference from histocompatability factors.

Major Factors:

In the systems described above, a total of six different tumors have been tested. No inhibition of the development or manifestation of tumor immunity could be detected as a consequence of C'5 complement deficiency. Whether this indicates that whole hemolytic complement activity is unnecessary for tumor immunity to be effective or whether a level of complement activity too low to detect by present methods is sufficient remains unsettled. In the light of present concepts of tumor immunity, the former possibility seems more likely, since it is generally believed that tumor immunity is a manifestation of celluls immunity which does not require complement to be effective. During the course of these experiments data was obtained which

suggest that the B10-D2 "old" and "new" strains are not fully syngeneic. If confirmed by studies in progress, it will cast doubt on several previous studies based on the assumption that these are syngeneic strains. Also, this may provide a useful tool for transplantation immunology in that histocompatability testing may be made more sensitive by the use of tumor rather than normal tissues for sensitization.

Significance to Bio-medical Research and Program of the Institute:

The apparent absence of need for complement in tumor immunity argues against an important role for humoral antibody in the manifestation of tumor immunity. When extrapolated to the problem of immunotherapy of human neoplasms, it suggests that passive antiserum therapy will not be effective, but that active induction of cellular immunity will be needed, either by active sensitization of the tumor-bearing individual's lymphoid systems or by intra-individual transfer of sensitized lymphoid cells. Both these latter methods pose greater problems than does the use of antiserum.

Proposed Course of Project:

No further studies are presently planned in respect of the role of complement, as the data from these experiments are quite clear. However, sufficient problems have arisen from attempts to use the tumor-amputation-challenge method in quantitative fashion that a more detailed study of the quantitative kinetics of the system is indicated. Some of the parameters to be evaluated are; (1) the relative roles of duration of tumor growth prior to amputation and size of immunizing tumor, (2) timing of amputation and subsequent challenge, (3) site and volume of challenge injection, (4) effect of the presence of clinically inapparent residual tumor at the site of amputation, (5) effect of the presence of microscopic pulmonary metastases. Also, efforts will be made to develop a satisfactory technique for quantitating rate of tumor growth in the early period after challenge before tumor volume measurements are complicated by necrosis and hemorrhage in the tumor mass.

Project Description:

Part III. Feasibility studies of immunotherapy for solid tumors

Objectives:

To test various modalities for passive transfer of immune substances or immunized cells with respect to their effect on the growth rate of established tumors in mice.

Methods Employed:

This project was inactive during the past year as efforts were directed towards development of more satisfactory model systems for testing.

Major Findings:

None.

Significance to Bio-medical Research and Program of the Institute:

While ample data exist to show clearly that the take and subsequent growth of tumor inocula can be prevented or inhibited by immunologic means, it is the ability to favorably alter the growth of the established tumor that is crucial to immunotherapy for human cancer. In complete syngeneic tumor-host systems, this has not been achieved by any method applicable to patients. We believe that laboratory demonstration of an effective anti-tumor immunotherapy should precede clinical trials.

Proposed Course of Project:

Using the newly available tumor-host systems mentioned above, efforts will be directed towards ascertaining first if continued growth of immunogenic tumor represents a form of persistent immunologic tolerance, insufficient immunization, or inadequate vigor of the effector mechanism in a maximally sensitized individual. The results of these studies will determine the later course of the project.

Project Description:

Part IV. Immunobiology of the lymphoid tissues in inbred mice with chemically-induced tumors

Objectives:

Earlier studies from these laboratories indicated that ablation of the regional lymph nodes did not alter the response to subsequent contralateral tumor inoculation of animals whose original tumor was amputated just prior to the regional lymphadenectomy. Recent reports from elsewhere have suggested that retention of the regional nodes for several weeks after amputation exerts a protective effect in respect of both local wound recurrence and distant metastases. However, these studies were done with homologous tumors and by relatively imprecise methods for evaluation of metastases. The current investigation was undertaken to find out whether regional nodes are indeed important in the control of metastases in a syngeneic tumor-host system known to be immunogenic.

Methods Employed:

A large number of female C57BL/6N mice were injected in the foot with a syngeneic methylcholanthrene-induced fibrosarcoma of known immunogenicity and proclivity for pulmonary metastases. When the tumors grew to 1.0 cm. in diameter or greater, the animals were randomly divided into 11 equal groups. One group was retained as untreated controls, 4 groups underwent

local amputation of tumor, one group underwent local amputation plus excision of the regional nodes, and two groups underwent a radical amputation which included the regional nodes as part of the specimen. The group treated with simple amputation plus node excision, one group which underwent simple amputation and one group undergoing radical amputation received an injection of viable tumor cells into the incision at the completion of the operation. About 10 days after the procedure, one of the simple amputation groups underwent the radical amputation and another early simple amputation group received the radical amputation plus excision of the regional nodes proximal to the site of radical amputation. Three previously untreated groups underwent either cimple amputation, radical amputation, or radical amputation plus proximal regional node excision. All animals were necropsied at time of death or when sacrificed one month after the date of the second procedure. Serial sections were made of all regional nodes from both operative and necropsy material. Lungs were examined carefully with a low-power lens and grossly suspicious area examined histologically.

Major Findings:

Since submission of this report last year, study of the large volume of histologic material has been completed. Full evaluation of the data in no way alters the preliminary conclusions that it is the time of tumor removal which is important rather than the type of procedure done, so long as complete tumor removal is accomplished. Presence or absence of regional nodes was unrelated to the eventual development of tumor from wound seeding done immediately after operation. Further reinforced by completing the histologic evaluation was our firm conclusion that metastatic disease in regional nodes of tumor-bearing mice can only be diagnosed by multiple sections of the node. Another finding worthy of inclusion is that among the wide variety of mouse tumors initially studied, to pick the one with the highest incidence of nodal metastases to use in this study, there was a very low incidence of nodal metastases in general among the tumors studied.

Significance to Bio-medical Research and Program of the Institute:

These data, plus the earlier studies, mentioned above, indicate clearly that for immunogenic murine sarcomas the regional lymph nodes are not of immunologic importance in determining the eventual outcome of the tumor-host relationship. The postulate that regional nodes should be left in situ when grossly free of disease in patients being operated on for tumor is untenable, and the classic concept of an en bloc resection of primary tumor plus regional nodes remains valid. The importance of the observation that murine neoplasms usually metastasize directly to the lungs without including the regional nodes is tantalizing, and no reasonable explanation is available.

Proposed Course of Project:

No further work is presently planned on this project, although concern still exists in regard to the problem of why mouse cancer doesn't metastasize to regional nodes. More work may be undertaken to seek an answer to this question

if testable hypotheses are developed.

Project Description:

Part V. Immunologic relationships among murine mammary tumor agents

Objectives:

To ascertain whether the mouse mammary tumor viruses indigenous to various strains of mice are immunologically related in a fashion similar to the polyoma virus strains.

Methods Employed:

Three tumor-host systems are used in this study: The C3H/HeN mouse carrying the classic C3H mammary tumor virus, RIII/An mice with their typical virus of long standing, and RIII/An mice with a high incidence of mammary tumors due to the C3H virus which was introduced into this strain by foster nursing methods. The C3H/HeN x RIII/An F_1 ($cfR3F_1$) hybrid is used for testing, since it carries no virus but will accept tumor transplants from any of the three systems mentioned. $CfR3F_1$ mice are given one of the three tumors and then the tumor-bearing leg is amputated. Seven to ten days later these mice are challenged with quantitative single-cell suspensions from one of the other tumors. Normal animals and mice given a second dose of the same tumor are used for controls. Tumor incidence following challenge are compared.

Major Findings:

Not much work has been possible in this project during the past year owning to the relative unavailability of RIII/An mice for production of the test animals used, the C3Hf/HeN x RIII/An F_1 hybrid. RIII/An mice are available only from the Jackson Laboratory or from Dr. H.B. Andervont, and have been in very short supply. However, the data obtained thus far indicate that these objectives of the project may well require alterations in methods, in that little or no immunity of any sort has been demonstrable by the transplantation resistance technique. The probable tolerance-inducing viremia, presumably a result of incubation with virus releasing tumor, remains an unsolved problem, as in some instances enhancement of tumor growth has occurred following tumor amputation and subsequent challenge.

Significance to Bio-medical Research and Program of the Institute:

If it can be shown that persistent viremia with an oncogenic virus causes tolerance and enhancement of tumor growth or dampens the effect of an anti-tumor immune response to an otherwise immunogenic tumor, the important consequence for immunotherapy of human virus-induced tumors is that it will be necessary to develop measures effective against both virus and tumor, since neither would be effective alone.

Proposed Course of Project:

Accumulation of sufficient animals to continue the experiments is now progressing more satisfactorily. Using methods developed in the Immunology Branch, NCI, for the isolation of cell-surface histocompatibility antigens, it is planned to attempt immunization with virus-free tumor antigen and compare the response to subsequent challenge with that exhibited by exposure to both tumor and virus as a result of tumor growth and subsequent amputation. Since the spleen of mammary tumor-bearing mice is infected with the virus, the role of viremia will be studied in mice receiving inocula of spleen cells from syngeneic tumor-bearing animals.

Project Description:

Part VI. Acquired immunological tolerance in carcinogenesis by the mammary tumor virus

Objectives:

Cancer specific antigens capable of eliciting a host immune response which specifically inhibits neoplastic growth have been demonstrated in all viral-induced neoplasms which have been adequately studied. Therefore, natural infection with an oncogenic virus would not be expected to proceed to tumor formation unless this host immune response has been inhibited in some manner. Perhaps the most likely mechanism for immune suppression by tumor viruses in nature is by the induction of specific immunologic tolerance to the viral-induced cancer specific antigen following neonatal infection with the oncogenic virus. The natural history of the mouse mammary tumor virus made this neoplasm an ideal model which could be used to test our hypothesis. The mammary tumor virus is present in high concentration in the milk of mouse strains carrying this virus; therefore, the newborn mouse is exposed to the virus from the moment it begins to suckle from its mother's breast. Its initial exposure is begun at a time prior to the onset of immunologic competence and continues daily until weaning. This situation might be expected to produce immunological tolerance to the mammary tumor virus itself, and perhaps to any viral antigens present on the cell membrane of tumor cells induced by this virus. Our previous work has suggested that mice congenitally infected with the C3H mammary tumor virus become immunologically tolerant as adults to a cancer specific antigen in mammary carcinomas induced by this virus.

Our present objectives are as follows:

1. To determine if acquired immunological tolerance is of importance in the development of mammary tumors in other strains of mice that develop a high incidence of spontaneous mammary adenocarcinomas, such as strain DD and the high mammary tumor sublines of the BALB/c strains.

2. To develop methods of abolishing the acquired immunological tolerance induced C3H mice by congenital infection with the mammary tumor virus.

3. To determine the antigenic specificity of the mammary tumor viruses from favious strains of mice developing a high incidence of mammary adenocarcinomas.

Methods Employed:

1. Our experimental method of determining the existence of immunological tolerance to mammary adenocarcinomas consisted of comparing the resistance against challenge with mammary tumor inocula of adult mice congenitally infected with the mammary tumor virus with the resistance to similar inocula of genetically identical mice freed of this virus by foster nursing on sublines of mice not infected with the mammary tumor virus. It is assumed that increased resistance against challenge with mammary tumor found in sublines of mice uninfected with mammary tumor virus is on an immunological basis if a total body irradiation greatly reduces the resistance of virus-free mice against tumor challenge but has little or no effect upon the response of mice congenitally infected with the mammary tumor virus.

2. We have attempted to abolish the immunological tolerance to spontaneous mammary carcinomas exhibited by mice congenitally infected with the mammary tumor virus. An attempt has been made to change the antigenic specificity of the tumor specific antigen (or antigens) on the mammary tumor cell by coupling the tumor cell with a heterologous antigen by bisdiazotization to heterologous proteins such as human gammaglobulin and hemocyanin. It is hoped that these heterologous proteins will act as a hapten to the tumor specific antigen and thereby change the immunological recognition of the tolerant mouse to the tumor specific antigen.

3. The antigenic specificity of the mammary tumor virus from various strains of mice is being investigated using sublines of virus infected and virus free mice from the same strain. The virus free sublines of mice are immunized with histoincompatable mammary tumors originating in other strains of mice, such as the A, DD, RIII and C3H. The tumor being allogenic is rejected, but the virus free mouse is exposed to both the mammary tumor virus and any virus specific tumor antigens in the mammary carcinoma cell. Serum from the immunized mice is then tested for antibodies against a mammary tumor arising in the virus infected subline of the same strain of mice in which the antiserum was produced. Since the antiserum is tested against isogenic mammary tumor, any serological reaction observed must be related to virus specific or tumor specific antigen in the mammary tumor. Immunological techniques currently being applied to the problem consist of immunofluorescence, quantitative complement fixation and antiglobulin consumption.

Major Findings:

1. The adult C3H/HeN mouse which is congenitally infected with the mammary tumor virus appears to be completely immunologically tolerant to spontaneous mammary adenocarcinoma induced by this virus. Studies with sublines of the BALB and DD strains of mice indicate that immunological tolerance also is produced by neonatal infection with both the DD and C3H strains of mammary tumor virus. Furthermore, DD strain of mammary tumor virus appears to be capable of inducing immunological tolerance to mammary tumors induced by the C3H strain of mammary tumor virus and vice versa. Thus it appears that acquired immunological tolerance may be of importance in the development of mammary tumors in many strains of mice.

2. All attempts to increase the antigenicity of spontaneous mammary tumors by coupling the tumor cell to the heterologous antigen have been unsuccessful, with one minor exception. Coupling of mammary tumors to hemolymph resulted in a very slight increase in antigenicity over that obtained by immunization with tumor cells alone.

3. Virus free mice immunized with repeated injections of histoincompatible mammary tumors have formed antibodies which react with isogenic mammary tumor cells by immunofluorescence, complement fixation and antiglobulin comsumption tests. Most of our data has been accumulated with an anti-serum produced in a C3Hf mouse and tested against a C3H/HeN spontaneous mammary tumor. Thus far, every mammary tumor induced by the C3H strain of mammary tumor virus (regardless of whether that tumor arose in a BALB/c, C3H, or RIII strain of mice) has been able to absorb the reactivity of the C3Hf antisera against the C3H mammary tumor. Furthermore, lactating mammary tissue from virus infected, but not from virus free mice has similarly been capable of removing this antibody activity. Therefore, it would appear that the antibodies being detected by these tests are antibodies against the mammary tumor virus itself or a virus specific antigen produced in both normal and tumor cells following infection with the virus.

Significance To Bio-medical Research and Program of the Institute:

Further studies will be necessary to evaluate the importance of acquired immunological tolerance in the natural history of other oncogenic viruses. However, if the ability to induce immunological tolerance proves to be an important prerequisite for effective carcinogenesis by oncogenic viruses in nature, then effective immunotherapy of virus-induced cancer will depend on the development of methods for breaking this acquired immunological tolerance to the viral-induced cancer antigens, rather than upon the production of cancer vaccines. A vaccine against breast cancer in C3H mice would not be expected to either prevent tumor development or retard its growth, since the mice that will develop breast cancer can not recognize

the tumor antigen as foreign because they are already immunologically 'blind' to this antigen.

Proposed Course of Project:

We feel that this work is of enormous importance to our understanding of the mechanism for viral oncogenesis in nature. Therefore, we plan to continue working on this problem in the manner outlined before.

Project Description:

Part VII. In vitro stimulation of autochthonous lymphocytes by the host system

Objectives:

To develop an in vitro test system for the measurement of the effects of human tumors on the metabolism of lymphocytes obtained from the tumor host at varying periods during the clinical course.

Methods Employed:

Peripheral blood lymphocytes from patients scheduled for excision of malignancies are cultured in media containing autologous, homologous and heterologous sera. A portion of sterile tumor is prepared both as cellular suspension and acellular extract. Tumor thus prepared is added to the lymphocytes in culture and at varying periods afterwards tritiated thymidine is added to the culture. The uptake of the isotope is measured in a liquid scintillation counter, and with radioautographs of the cultured cells made simultaneously, the percentage of cells showing uptake of the isotope is calculated. Lymphocytes from normal volunteers and muscle tissue from the patients serve as controls for the patient's cells and the tumor respectively.

Major Findings:

On exposure to the host tumor cells in suspension and to tumor extracts, lymphocytes from patients having a variety of tumors show significantly increased uptake of tritiated thymidine and radioautographs show increased percentage of blast stage lymphocytes with tritiated thymidine uptake. This reactivity of the patients' lymphocytes does not occur on exposure to autochthonous muscle. This reaction regularly occurs in autologous and heterologous sera, but irregularly so in homologous sera. Studies of the effects of several substances commonly used in lymphocyte isolation for culture--gum arabic, methyl cellulose, iron, and dextran--show that all may stimulate lymphocytes in culture, therefore, methods of separation not involving the addition of such substances have been developed.

Significance to Bio-medical Research and Program of the Institute:

This reaction of lymphocytes to host tumor appears to be idential with the

effect on lymphocytes of antigens to which the cells have been previously exposed. In such instances, production of antigen-specific antigody by th lymphocytes results. This experiment suggests, therefore, that the tumors studied thus far have envoked an immunological reaction by the host and that the lymphocytes are the mediators of this reaction. The magnitude and duration of this immune response to cancer may be measureable by these means, and the effects of various treatments on hosts response, such as chemotherapy and immunotherapy, may be assessed.

Proposed Course of Project:

Portions of the tumor suspensions and extracts studied are preserved by freezing. At intervals of months to years after removal of the tumor, the in vitro reactions of the patient's lymphocytes to the tumor will again be measured. A correlation with the patient's clinical course will be attempted, i.e. persistance of lymphocyte reactivity might be correlated with residual tumor in the patient, a loss of reactivity may be due to complete removal of all tumor at the operation assuming that the patient is in good health at the time of repeat study. Isolation of the specific portion of the tumor cell inducing the lymphocyte reaction observed will be attempted. If it is successful, additional studies will be made in an attempt to discover whether the stimulating factor is individual or specific by observing its effects on lymphocytes from patients with similar and dissimilar tumors. The object of these future experiments will be to discover a possible use for the lymphocyte-stimulating cell fraction in immunotherapy of patients with cancer.

Project Description:

Part VIII. Immunologic studies with human neoplasms

Objectives:

Cancer specific antigens have been found in every adequately studied viral or carcinogen induced animal neoplasm. Initially, most of these antigens were demonstrated by their ability to induce tumor specific transplantation resistance following pre-sensitization of the isogenic host by temporary tumor growth or by pretreatment with irradiated tumor cells. However, these tumor-specific antigens have now been demonstrated by a variety of immunological techniques including cytotoxicity, complement fixation, gel diffusion and immunofluorescence reacton. It is logical to assume that human neoplasms contain cancer-specific antigens similar to those found in animal tumors, but there is presently no unequivocal evidence for the existence of such antigens. An initial step in proving the existence of cancer-specific antigens in human neoplasms would be the demonstration of antibodies in the serum of cancer patients which react specifically with autologous tumor tissue.

The purpose of this study was to determine whether antibodies could be

demonstrated by immunofluorescence techniques in the serum of cancer patients which react with autologous or homologous tumors.

Methods Employed:

Tumor tissue is removed from the surgical specimen as soon as possible after its removal from the patient. Tumor tissue is then studied by two different immunofluorescent techniques. Tumor imprints are made on glass slides and stored in a liquid nitrogen refrigerator until used in the direct or indirect immunofluorescent test against acetone fixed tumor. A single cell suspension of living and tumor cells prepared for use in the indirect membrane immunofluorescent test described by Miller. Where possible normal tissues from the same organ will be similarly treated and tested.

The patient's own serum, homologous serum from other patients with the same neoplasms, and normal sera are tested against both the acetone fixed and living cell preparations.

Initially most of our studies have been performed with melanomas, sarcomas, and colonic neoplasms.

Major Findings:

Autologous and homologous sera from 27 patients with malignant melanoma and 25 normal sera have been tested for reactivity by immunofluorescence techniques against 7 different melanoma. Anti-melanoma antigodies have been demonstrated in all the 7 patients whose sera were tested against their own melanomas by the indirect immunofluorescence techniques against suspensions of living cells or acetone fixed tumor imprints. Anti-melanoma antibodies were found in 61% of the tests of homologous melanoma sera and in 20% of the tests of homologous normal sera against these melanomas. The antibody nature of the immunofluorescence reactions have been confirmed by blocking and absorption studies. These data suggest that most malignant melanomas contain a common tumor antigen which is immunogenic in patients with this neoplasm.

Similar studies with human sarcomas and human colorectal neoplasms have yielded almost identical findings.

Significance to Bio-medical Research and Program of the Institute:

Further studies will be necessary to definitely establish that these immunofluorescence studies are identifying a truly tumor-specific antigen by the reactivity between autologous serum and tumor tissue. However, all data presently available is consistent with this hypothesis. Furthermore, the detection of a common tumor antigen shared by many neoplasms of the same histological type has important implications for the etiology of these tumors. It is well known that all animal neoplasms induced by the same oncogenic virus contain similar tumor-specific antigens. Carcinogen induced

neoplasms on the other hand contain individually specific tumor antigens. This sharing of a common tumor antigen among human neoplasms, therefore, offers presumptive evidence of their viral etiology.

Proposed Course of Project:

We plan to continue these studies in an attempt to identify the different types of antigen which can be found on human neoplasms. Other immunological techniques such as immune cytolosis, complement fixation, and antiglobulin consumption will be applied to the detection of these tumor antigens.

Attempts are currently being made to isolate a tumor virus from these human neoplasms.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Chretien, P.B., Behar, R.J., Kohn,Z., Moldana, G., Miller, D.G. and Lawrence, W.: The canine lymphoid system. A study of the effects of surgical removal. Anat. Rec. 159: 5-16, Sept. 1967

Fisher, J.C. and Hammond, W.G.: Parabiotic transfer of tumor-specific immunity. Cancer 21: 160-164, Jan. 1968.

Hammond, W.G., Fisher, J.C. and Rolley, R.T.: Tumor-specific transplantation immunity to spontaneous mouse tumors. Surgery 62: 124-133, July 1967.

Irvin, G.L. and Carbone, P.P.: Immunosuppression with lymph depletion in man. Surg. Gynec. Obstet. 124: 1283-1287, June 1967.

Irvin, G.L., Eustace, J. and Fahey, J.: Enhancement activity of mouse immunoglobulin classes. J. Immunol. 99: 1085-1091, Dec. 1967.

Serial No. NCI-3809

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Serum Fractions Influencing Cell Growth

Previous Serial Number: Same

Principal Investigators: Dr. Robert H. Johnson, Dr. Willis E. Brown, Jr.

Other Investigators: Dr. Alfred S. Ketcham, Dr. Fitzhugh X. Mullins, Dr. Stanley Schwarz, Dr. Seymour Perry, Dr. Louis B. Thomas, Dr. Richard Wurtman

Cooperating Units: Surgery, Medicine, and Pathologic Anatomy Branches, NCI; Laboratory of Clinical Science, NIMH

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1
Professional:	1
Other:	0

Project Description:

Part I. Intensive chemotherapy with methotrexate utilizing regional protection with citrovorum factor

Part II. Studies on arterial infusion chemotherapy

Project Description:

Part I. Intensive chemotherapy with methotrexate utilizing regional protection with citrovorum factor

Objectives:

The purpose of this project was to determine whether small doses of citrovorum factor administered via the hypogastric arterial infusion would selectively protect the pelvic marrow against the effects of systemically administered methotrexate. This was done in an attempt to find a method to increase the dose of methotrexate that could be safely given to patients, either regionally or systemically. In addition, the possible beneficial effects of continuously administered non-absorbable intestinal antibiotics in lessening gastro-intestinal toxicity was studied.

Methods Employed:

Rhesus monkeys are utilized. Methotrexate was given by continuous intravenous infusion for seven days, while small doses of citrovorum factor (1 1/30 to 1/10 of the methotrexate dose) was given concomitantly by continuous hypogastric arterial infusion. At the same time intestinal antibiotics were given also by continuous naso-gastric infusion. The effects on formed blood elements and body tissues, especially marrow, was studied.

Major Findings:

In only a few of 50 animals would it be clearly shown that the "regionally" protected marrow was less affected than distant marrow. When regional protection occurred it was often striking, but quite unpredictable in occurrence. The protection of the gastro-intestinal tract by intestinal antibioties, however, was very impressive.

Significance to Bio-medical Research and Program of the Institute:

The concept of regional protection of the marrow probably is not useful clinically. However, the use of intestinal antibiotics in patients undergoing intensive and prolonged chemotherapy should be valuable in preventing gastro-intestinal toxicity.

Project Description:

Part II. Studies on arterial infusion chemotherapy

Objectives:

1. To assess the toxicity of internal carotid infusion of methotrexate.

2. To evaluate the protection against internal carotid infusion of methotrexate afforded by concomitant regional arterial infusion of citrovorum factor and gastro-intestinal infusion of antibiotics.

3. To evaluate pathologic effects of internal carotid infusions of methotrexate upon normal brain and upon brain tumors or tumors in the brain.

Methods Employed:

Internal carotid arteries of 3-4 Kg Rhesus monkeys are infused continuously with methotrexate at the dose of 1 mgm/Kg/24 hours. Citrovorum factor is infused concomitantly into the hypogastric artery to protect the iliac marrow and antibiotics are infused into the gastro-intestinal tract to protect the mucosa. The general status, death time, white blood cell counts and extent of gastro-intestinal toxicity are observed.

Major Findings:

Infusion of methotrexate at this dose level produced death in six of six monkeys infused. Leukopenia in five of six monkeys and severe diarrhea in three of six monkeys were encountered. Protection was afforded by citrovorum factor. The dose of citrovorum factor employed (1/15th the methotrexate dose) was far less than that dose usually employed clinically. The gastro-intestinal antibiotics reduced the degree and incidence of diarrhea. Fatalities were reduced from 100% to 50%.

Final review of specimens from infused monkeys revealed that this infusion system did not produce a lesion attributable to methotrexate per se.

Significance to Bio-medical Research and Program of the Institute:

This work extends previous studies on regional protection by the metabolite during systemic (intravenous) infusion of the antimetabolite. The infusion of the antimetabolite (methotrexate) into the arterial bed of a tumor is expected to increase the effectiveness of the drug, a conclusion based on rather wide clinical experience. In addition, the regional uptake of the metabolite (citrovorum factor) is expected to minimize the protective action of the metabolite upon a tumor. If this premise is correct, the therapeutic index of arterial chemotherapy may be significantly increased.

Proposed Course of Project:

Additional studies on the pathologic effects of this infusion-protection system are planned. Several approaches to the development of an intra-cranial tumor in primates are under investigation. Preliminary work suggests that choriocarcinoma grows in Rhesus monkey brain. We plan intensive study of this and related intra-cranial tumor systems. When the growth of chorio-carcinoma in the monkey brain is well defined, we plan to study the response of the tumor to internal carotid methotrexate infusion and related topics.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Gittes, R.F.: Thyrocalcitonin: A new look at urinary calcium homeostasis. Urologic Internationalis 22: 285-300, 1967.

Gittes, R.F., Wells, J.H. and Irvin, G.: A new role for the parathyroid gland in calcium homeostasis. J. Urol. 97: 1082-1089, June 1967.

Serial No. NCI-3810

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Surgical Physiology of the Genitourinary Tract; Laboratory and Clinical Studies Related to this Anatomic Area

Previous Serial Number: Same

Principle Investigator: Dr. Elwin E. Fraley

Other Investigators: Dr. D. F. Paulson, Dr. A. S. Rabson, Dr. J. Doppman, Dr. B. Jacobs, Dr. S. Epstein, Dr. B. Lucas, Dr. J. DeKernion, Dr. D. Brandes, Dr. R. Johnson, Dr. M. Kirschner, and Dr. P. Mora

Cooperating Units: Department of Diagnostic Radiology, Department of Pathologic Anatomy, Department of Clinical Anesthesiology, NCI Endocrinology Branch, Department of Pathology of the University of Maryland, Macromolecular Biology Section, GL&C, NCI

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1-1/2
Professional:	1
Other:	1/2

Project Description:

Part I. Studies on Intrarenal Obstructions

Part II. Studies on SV40 Transformed Prostate Tissue

Part III. Molecular Biology of the Kidney

Part IV. Development of an Artificial Urinary Sphincter

Part V. Electrical Stimulation of Bowel Substitutes for Bladder

Part VI. Studies of the Effect of Methoxyflurane (Penthrane) on Postoperative Renal Function

Part VII. Clinical Studies on Patients with Anorchidism

Part VIII. Protection Against Radiation Damage to the Kidney by Intra-arterial Injection of Epinephrine

Project Description:

Part I. Studies on intrarenal obstructions

1. To develop methods for studying abnormalities of emptying in the proximal collecting system.

2. To develop surgical techniques for correcting disorders of peristalsis in the proximal collecting systems.

3. To develop new techniques for the correction of other forms of intrarenal disease such as arteriovenous aneurysms, calyceal diverticula and segmental pyrolonephritis.

4. To study the effects of small amounts of residual urine of the proximal collecting system and renal function. Also to investigate the relationship between minute quantities of residual urine and the initiation and perpetuation of both calyceal stones and urinary tract infections.

Methods Employed:

Patients have been thoroughly evaluated using standard urological and radiographic techniques. However, special attention has been paid to cine radiography of the upper urinary tract. Recently, also, radioactive scanning techniques employing the (gamma) scintillation photoscanner have proven particularly interesting. Techniques for improving the visualization of the proximal collecting system using cine radiography and arteriography are currently under investigation. A new instrument is being developed with the cooperation of the American Cystoscope Makers and Company for visualizing the inside of calyces and infundibula at the operating table. Also new techniques are being developed in cooperation with the Department of Radiology for determining the relationship between the renal vessels and the infundibula preoperatively.

Major Findings:

Thus far it has been shown that renal vessels can compress the infundibulum--particularly the superior infundibulum--and thus cause obstruction of the calyx major. This obstruction probably causes nephralgia from overdistension of the calyx and its surrounding smooth muscle. In addition, such obstruction produces urine stasis in the calyx. We know that stasis in other parts of the urinary tract may perpetuate urinary tract infection and cause stone formation. Three of the patients we have studied have had urinary tract infections which cleared following correction of their intra-renal obstruction. Thus far, we have studied 9 patients with vascular obstruction of the superior infundibulum (seven adults and two children).

Significance to Bio-medical Research and Program of the Institute:

The techniques that have been evolved to correct vascular obstruction of the infundibulum are applicable to other intrarenal abnormalities such as renal stones and small tumors in solitary kidneys. In addition, it is hoped that this work will stimulate new thinking of possible conservative, kidney preserving procedures for the treatment of various other forms of intrarenal disease (e.g. calyceal diverticula, arteriovenous aneurysms and segmental pyelonephritis).

Proposed Course of Project:

Patients will continue to be studied as they become available. The projects related to the study of intrarenal obstruction will be completed.

Project Description:

Part II. Studies on SV40 transformed hamster prostate tissue

Objectives:

1. To develop an experimental tumor that closely resembles human cancer of the prostate.

2. To use this model system to examine the effects of hormones on growth of a prostatic neoplasm.

3. To clarify the relationship between prostatic neoplasms and hyper-acidphosphatemia.

4. To study the molecular organization of highly malignant, rapidly growing cells.

Methods Employed:

Prostatic tissue was obtained from Syrian (golden) hamsters and separated into dorsal-lateral and ventral-lobe portions. It was then minced into 1 mm. explants and used to establish *in vitro* explant cultures. When the cells began to migrate from the explants on the second or third days, one culture bottle each of the dorsal-lateral and ventral-lobe tissue was infected with a $10^{7.9}TCID_{50}$ (tissue culture infective dose, 50% effective) of SV40. The second bottle of these tissues was kept as a virus-free control. Cultures were observed daily for evidence of transformation. Twenty-two days after infection, all bottles were sub-cultured by trypsinization and transferred to larger bottles. After fifteen days of growth in the larger bottles, the transferred cells were scraped from the bottles and injected subcutaneously into adult male hamsters. All animals developed palpable tumors within 15 to 40 days. Tumors have subsequently been transplanted through more than 20 transplant generations in non-radiated hamsters. Tumors have grown rapidly and killed host animals between 14

and 21 days after transplantation.

The morphology of this tumor has been studied with the light microscope. Histochemical electronmicroscopic studies are currently being carried out.

Tartrate inhibitable serum acid phosphatase (TIAP)* has been studied employing standard assay techniques. Also, acid phosphatase (TIAP) has been isolated from the tumor cells by differential ammonium sulfate precipitation.

Studies on the molecular biology of this tumor have been carried out using standard techniques for this purpose. Many of the techniques are mentioned and outlined in Part III of this report.

Major Findings:

Hamsters prostate tissue has been transformed in vitro. When the transformed cells are injected into the animals, rapidly growing, malignant tumors are formed. This tumor resembles human cancer of the prostate in the following ways: (1) The tumor grows rapidly and metastasizes widely; (2) animals bearing the tumor have elevated serum TIAP levels; (3) the growth rate of the tumor is suppressed by estrogen and serum TIAP levels in tumor-bearing animals are normalized by estrogen.

At the macromolecular level, the classes of polyribosomes in these malignant cells have been surveyed. The polyribosomes exist mainly as single and oligoribosomes with very few heavy, rapidly sedimenting ribosomes. Thus the polyribosome profile from this tumor is rather unique. All classes of ribosomes seem to be capable of synthesizing protein although specific activity (CPM/unit optical density) is less for the dimers and monosomes than for the larger oligoribosomes. Unlike most other animal and plant cells, the percentage of total cellular RNA that exists as membrane-bound ribosomes is extremely small (approximately 5%). Also the amount of non-sedimenting RNA (tRNA, mRNA, etc.) is higher in the tumor cells than in normal cells.

Further detailed studies have been carried out in individual ribosomes from this tumor. The individual ribosomes have been isolated from various segments of the polyribosome profile. We have discovered that these ribosomes respond in an unusual way to 0.001 M EDTA (which binds all available magnesium in our system); that is, the ribosomes completely dissociate to 28^S and 18^S RNA sub-units and ribosomal protein. All previously studied ribosomes (plant, bacterial and mammalian) have required more vigorous chemical treatment to separate their ribosomal RNA from its ribosomal proteins. Detailed studies of the chemical composition of these ribosomes are underway in an attempt to determine the basis of this unusual phenomenon.

* TIAP generally accepted as indicating equivalent of prostatic acid phosphatase.

Significance to Bio-medical Research and Program of the Institute:

We feel that it is particularly significant that this experimental tumor resembles its human counterpart in many ways. Although further study of this model system may not measurably increase our understanding of human cancer of the prostate per se, there is reason to hope that continued investigation of this neoplasm may provide important and new information in the following three areas: 1) the relation between prostatic neoplasms and hyperacidphosphatasemia; 2) the mechanism of action of estrogen in inhibiting the growth of prostatic neoplasm; 3) the molecular organization of rapidly growing, malignant cells.

Proposed Course of Project:

We plan to continue the studies at the subcellular level of the macromolecular assemblies for proteins, the various aspects of RNA and protein synthesis and the structure of the individual tumor ribosomes.

We also are interested in how estrogen influences protein synthesis and growth in these cells. Furthermore, detailed studies to the effect of testosterone, progesterone and combined testosterone-estrogen preparation on the growth of this tumor are planned. Also using the techniques of electron-microscopy and histochemistry, we plan to investigate how estrogen normalizes serum prostatic acid phosphatase in tumor-bearing animals.

Project Description:

Part III. Molecular Biology of the Kidney

Objectives:

The purposes of this study are as follows: 1. To develop new techniques for characterizing the macromolecular changes in renal cells under various experimental situations. 2. To characterize subcellular changes in ischemic renal cells following acute obstruction of the ureter. 3. To document biochemical changes in the opposite kidney following renal artery ligation, renal pedicle ligation, and nephrectomy. Hopefully, these latter studies will provide information about early cellular reorganization in the kidney prior to compensatory hypertrophy.

Methods Employed:

Functional analysis of the subcellular components of the kidney requires initial homogenization to disrupt the cells. Homogenization must be performed gently so as not to destroy the integrity of subcellular particles. Various cell organelles can be separated for study by ultracentrifugation of the homogenates on sucrose gradients. Studies using radiotracers to investigate protein synthesis and ribonucleic acid (RNA) metabolism are also being carried out. Standard techniques for identifying various subcellular particles in sucrose gradients have been employed.

Major Findings:

The results of our studies thus far are as follows:

1. Acute ureteral ligation--the mouse kidney has been used to study the effects of acute ureteral ligation. Morphological changes following acute ureteral obstruction with the light microscope have been documented. In addition, cells of kidneys obstructed from one day to three weeks have been homogenized and the subcellular particles have been examined. These studies show that there is an initial biochemical response to obstruction which consists of an increase in RNA synthesis and an increase in a number of polyribosomes per cell (determined by RNA/DNA ratios). The gradual increase in RNA synthesis and numbers of polyribosomes is progressive and reaches a peak at approximately 3-4 days. Thereafter, polyribosomes undergo a gradual decrease but even up to two weeks after obstruction, the structure polyribosomes are well preserved in the obstructed kidney at a time when it is probably irreversibly damaged.

Although ribosomal RNA synthesis seems to decrease progressively after three days of ureteral obstruction mRNA synthesis appears to be unaffected (possibly the synthesis of certain classes of mRNA is actually increased). Further studies of the effect of acute ureteral obstruction on the synthesis of various classes of RNA are in progress.

2. Studies on renal ischemia--mouse kidneys were made ischemic by complete ligation of the renal artery and renal pedicle. With complete ischemia of the kidney, there is a gradual decrease in amount of polyribosomal material per cell. In previous studies we have determined that the mouse kidney can tolerate ischemia for 1-1½ hours. After 60 minutes of total ischemia polyribosomes are still very well preserved. We are attempting to determine at the present time whether these ribosomes are capable of protein synthesis. Also under investigation is the mechanism whereby the gradual loss of polyribosomes occurs in the ischemic cell.

3. Studies of the contralateral kidney--changes in the polyribosomes to the contralateral kidney immediately following ligation of the ipsilateral renal artery, renal pedicle, and nephrectomy were studied. Animals were again Charles River strain mice. Thirty minutes after pedicle ligation or nephrectomy there is a steady decrease in both the membrane bound (DOC-treated) and free heavy, rapidly-sedimenting polyribosomes and an increase in single ribosomes in the contralateral kidney. These changes were maximum after 2 to 3 hours. It is of particular interest that similar changes did not occur following acute high ureteral ligation.

Observed polyribosome changes were similar to those which are produced by ischemia, except that there was never complete disappearance of the heavy polyribosomal material such as that accompanying irreversible ischemic cell damage. Additional experiments have shown that the loss of polyribosomes is not due to the activation of a nucleolytic system. Studies are currently

underway to determine whether the observed changes in the polyribosomes can be accounted for by a change in the rate of messenger RNA (mRNA) synthesis.

Significance to Bio-medical Research and Program of the Institute:

Although many investigators have documented the effects of hypoxia, stress and shock on overall renal function almost nothing is known about the subcellular biochemical changes that accompany shock and stress, much less the simpler situation of hypoxia alone.

If precise biochemical criteria can be established to distinguish between reversible and irreversible ischemic cell damage, then hopefully they can be applied in assessing viability of stored tissue and organs to be transplanted. In addition, the same type of criteria may be defined so that they can be used to determine whether a kidney has been irreversibly damaged by ureteral obstruction.

Also, it is hoped that new information will be obtained on the initial phase of cellular reorganization in preparation for compensatory renal growth.

Project Description:

Part IV. Development of an artificial urinary sphincter

Objectives:

At the present time, there is no adequate treatment for total urinary incontinence. Therefore, the object of this study is to develop a simple, reliable means for treating total urinary incontinence. This device will have its greatest use on patients with incontinence following radical perineal prostatectomy, but it may also be used to treat patients who are incontinent secondary to a congenital malformation such as myelomeningocoel.

Methods Employed:

The artificial sphincter will consist of two parts: there will be a urethral collar which contains an inflatable balloon and a control device that will be activated by manual pressure. The control device will act to inflate and deflate the collar surrounding the urethra. Hopefully the device will operate on the principles of a simple hydraulic system. The entire unit will be burried subcutaneously. The control unit will be placed just below the belt line and the collar will surround the bulbous urethra in the male and around the bladder neck in the female.

Thus far the device is still in the developmental stage. Progress has been made in the last year on the design of the control device, and a design for the collar has been finalized.

Major Findings:

Using a prototype device in dogs we have determined that one can compress the dog's urethra with pressure sufficient to interrupt urine flow without causing necrosis of the urethra. External pressure of 8 to 10 mm. applied circumferentially appears to be sufficient to interrupt the passage of urine.

Significance to Bio-medical Research and Program of the Institute:

If this device can be perfected, it will provide for the first time a simple reliable means for treating total urinary incontinence of varying etiologies. Also, if the principles of this device can be worked out, they may be applicable to similar problems in other parts of the body. For example, we are now investigating the possibility of developing an artificial sphincter for the use in the lower bowel to control fecal incontinence.

Proposed Course of Project:

It is hoped that there will be steady progress in the development of this device. The next stage will involve testing a prototype in animals. Clinical trials are planned following further animal experimentation.

Project Description:

Part V. Electrical stimulation of neurogenic bladders (clinical) and bladder replacements (laboratory)

Objectives:

The purpose of this project is twofold: First, we are continuing to evaluate the feasibility of using the currently available radio-linked bladder stimulators in patients; secondly, the possibility that bladder substitutes (bowel) can be made to empty by electrical stimulation is being explored.

Major Findings:

1. Clinical--Thus far we have had only one patient for implantation of a stimulator. The results in this case have been only partially successful, but it is probably to early to make a final judgment. Other patients are being evaluated for the possible use of a stimulator.

2. Laboratory--We have thus far succeeded in creating an experimental preparation in dogs consisting of an animal that has had his bladder completely removed and a piece of bowel substituted for the bladder. We have demonstrated, using a rather crude stimulator, that the bowel substitute for the bladder can be made to contract rather effectively.

Significance to Bio-medical Research and Program of the Institute:

At the present time there is no solution for the problem of neurogenic bladder. This is a common problem in a postoperative cancer patient as well as others with vesical dysfunction on the neurological basis. We are in hopes that a

combined clinical and laboratory approach may help to solve some of the problems relating to neurogenic vesical dysfunction.

Proposed Course of Project:

The studies outlined above will be continued in both the laboratory and in patients.

Project Description:

Part VI. Studies on the effects of methoxyflurane (penthrane^{r}) on postoperative renal function

Objectives:

It has recently been reported that Penthrane^{R} causes renal damage, producing a defect whereby the kidney can no longer concentrate urine. Patients who thus affected excrete very large quantities of dilute urine in the post-operative phase and this seriously complicates their management. This syndrome closely resembles nephrogenic diabetes insipidus.

Methods Employed:

A detailed protocol for a prospective study in 30 to 50 patients has been set up for the cooperation of the Department of Anesthesia. These patients will be studied in detail to see if we can accurately determine the incidence and nature of the so-called "Penthrane^{R} lesion".

Major Findings:

The study has just been initiated and thus far there are no major findings to be reported.

Significance to Bio-medical Research and Program of the Institute:

If we can establish that if Penthrane^{R} does indeed injure the kidney and further that this injury occurs frequently then possibly the anesthetic agent should be withdrawn from the market.

Proposed Course of Project:

We plan to continue the studies as outlined above.

Project Description:

Part VII. Clinical studies on patients with anorchidism

Objectives:

The object of this particular study are several fold. First, we would like

to study the endocrinological aspects of these patients. Second, we would like to develop a logical protocol for clinically evaluating patients with anorchidism. Third, as a result of this study we would like to perfect radiographic techniques for locating undescended testicles.

Methods Employed and Major Findings:

Thus far we have evaluated two patients with anorchidism. These patients have been studied medically and one has been explored surgically. Anorchidism was proved beyond a doubt in this patient even though he has a source of peripheral androgens--thus may represent a new clinical syndrome.

One additional patient has been studied endocrinologically and is awaiting surgical exploration.

Significance to Bio-medical Research and Program of the Institute:

If we can develop a logical plan for work-up in these patients, it will be an immense contribution. In addition, we may need to revise our thinking about the possible role of the adrenals in contributing androgens to the peripheral blood. Also, hopefully, techniques will be developed for locating undescended testicles which will have application in patients with unilateral cryptorchidism and not necessarily anorchidism.

Proposed Course of Project:

We will continue to study these patients and have sent out an announcement expressing our interests in this problem.

Project Description:

Part VIII. Protection against radiation damage to the kidney by intra-arterial injection of epinephrine

Objectives:

To develop a method for radioprotection of the kidney.

Methods Employed:

A red Kifa catheter was introduced into the renal artery by the Seldinger technique via the femoral artery. Epinephrine (1.3 micrograms in 2 cc. of solution per minute) was then infused with a sigma motor pump into the renal artery for 5 minutes, to produce hypoxia of one kidney. Next, without interruption of the infusion, 1,500 rads per minute (cobalt 60; 10 x 20 posterior field) were delivered to both kidneys.

At three months, the abdomens of the dogs were entered and both ureters were catheterized for timed urine collection.

Methods Employed:

In 4 of the 5 dogs surviving for the 3 month period it was noted that the unprotected (contralateral) kidney excreted no urine and was atrophic; weighing ¼ to ½ as much as the epinephrine treated kidney. Severe tubular degeneration and interstitial fibrosis were present in the non-perfused kidneys. Rare foci of minimal fibrosis was found in the epinephrine treated kidneys which excreted urine normally. The serum creatinine remained normal in the 4 successful test animals. The failure in the fifth test animal was most likely due to unrecognized dislodgement of the renal artery catheter before or during radiation treatment.

Significance to Bio-medical Research and Program of the Institute:

It is felt that perfection of this technique might allow intensive radiation treatment of the upper abdomen while preserving renal function. Furthermore, this technique is applicable to the transplanted kidney to decrease local damage from radiation rejection crisis and treatment.

Proposed Course of Project:

To study the physiological response of the transplanted kidney during perfusion and radiation.

Honors And Awards:

1. Drs. Elwin E. Fraley and David F. Paulson: First Prize: Laboratory Research Essay Contest of the American Urological Association. Awarded for studies of the SV40 induced experimental prostatic tumors. Paper to be presented at the annual meeting of the A.U.A. in May, 1968 to be held in Miami, Florida.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Behrens, M. and Holland, J.M.: Periureteritis plastica: Report of a case following staphylococcal infection. J. Urol. 97: 829-832, May 1967.

Fraley, E.E.: Surgical correction of intrarenal disease I. Obstruction of the superior infundibulum. J. Urol. 98: 54-64, July 1967.

Fraley, E.E. and Haverstadt, D.B.: Ruptured Ureter: An unusual complication of an ileostogram. J. Urol. 97: 1017-1018, June 1967.

Fraley, E.E. and Malt, R.A.: Specific intracellular defect in hypoxia. Surgery 62: 221-226, July 1967.

Halverstadt, D.B. and Fraley, E.E.: Avulsion of the upper ureter due to blunt trauma. Brit. J. Urol. 39: 588-593, 1967.

Leadbetter, G.W. and Fraley, E.E.: Surgical correction of total urinary incontinence. J. Urol. 97: 869-873, May 1967.

Nebesar, R.A., Pollard, J.J. and Fraley, E.E.: Renal vascular impressions Incidence and clinical significance. Amer. J. Roentgen., Radium Therapy and Nuclear Med. 101: 719-727, Nov. 1967.

Steckel, R.J., MacLowery, J.D., Holland, J., Paulson, D.F. and Johnson, R.: The Seldinger catheter in radiation therapy. Radiology 89: 332-335, Aug. 1967.

Weems, W.S., Washington, J.A. and Holland J.M.: The effects of acute and chronic renal artery constriction on the urinary oxygen tension in dogs. Surg. Forum XVIII: 496-498, 1966.

ARTICLES IN PRESS:

Fraley, E.E.: A book review of "Atlas of urological surgery" by Philip R. Roen. (New Eng. J. Med.).

Fraley, E.E.: Dismembered infundibulopyelostomy: An improved technique for correcting vascular obstruction of the superior infundibulum. (J. Urol.).

Fraley, E.E. and Ketcham, A.S.: Teratoma of the testis in an infant: Report of a case. (J. Urol.).

Fraley, E.E. and Paulson, D.F.: Morphological and biochemical studies of SV40 transformed prostatic tissue. (J. Urol.).

Serial No. NCI-3811

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Description: Laser Energy Studies on Normal and Malignant Tissues

Previous Serial Number: Same

Principal Investigators: Drs. Robert C. Hoye and Alfred S. Ketcham

Other Investigators: Grant C. Riggle, B.S., Drs. Louis B. Thomas, Kenneth B. Gray, Mr. Woodrow Stotler, George Weiss,PhD and Dr. Frederick R. Eilber

Cooperating Units: Biomedical Engineering & Instrumentation Branch, NIH; Eastman Kodak Company, U.S. Army Missile Command, Huntsville, Alabama; Department of Pathologic Anatomy,NIH; Chemotherapy Branch, NCI and Biometry Branch, NCI.

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1 1/2
Professional:	1 1/2
Other:	1/2

Project Description:

Part I. The destruction of normal liver and intra-abdominal tumor implants with laser energy, anatomical, pathological, and physiological consideration.

Part II. Quantitation of some physical parameters associated with the laser tissue interaction.

Part III. The chronic effects of the laser tissue interaction.

Part IV. Pathophysiologic studies of the effects of laser radiation upon normal and regenerating hepatic tissue in the rat.

Part V. The study of the intra-structure of cells at a distance from the laser tissue interaction.

Part VI. The clinical application of laser energy.

Project Description:

Part I. The destruction of normal liver and intra-abdominal tumor implants with laser energy, anatomical, pathological, and physiological considerations.

Objectives:

The monochromatic coherent properties of laser light make it possible for this instrument to vaporize any known substance. It is the object of this study to determine the effect of high energy neodymium laser light (10,600 A) on living tissue, both normal and malignant. Since its destructive potential for most substances is high it was hoped that this instrument would provide the physician with a means of killing large areas of cancer tissue in a controlled fashion. This project was started in 1963 and has continued. During the past year its main object has been to observe and describe the pathological effects on tissue and the thermodynamic properties involved.

Methods Employed:

The liver of the experimental animal was judged to be the organ most ideally suited for this study. Since it was large, it was homogenous, and was protected from external environmental influences. Changes in the penetration and absorption of laser energy in the area treated should be minimal. The Vx2 tumor in the rabbit liver was selected as an appropriate model for studying the effect of laser energy on tumor tissue. The growth characteristics and the appearance of the Vx2 tumor are similar to many human tumors.

Two lasers were used in these studies. One consisted of a neodymium rod, 1 inch in diameter by 32 inches in length and cooling was accomplished by liquid nitrogen. The second laser had a neodymium rod, 3/4 inch in diameter by 36 inches in length and cooling was accomplished with circulating deionized water. Each laser had an energy output at 1,000 joules per pulse in a time length of 2 to 2.5 milliseconds. The energy density on the target was varied by regulating the charge on the compacitor bank of the laser in combination with varying the distance of the target from the focusing lens.

Histological studies of the laser effects on normal liver tissue were made on 3-month old New Zealand white rabbits. The liver was positioned at right angels to the incident laser pulse. The animals were killed from 2 hours to 21 days post-laser and the histological sections routinely studied with hematoxylin and eosin, PAS, Diastase-PAS, Wylder's stain for reticulum, Masson Trichrom stain for connective tissue, Gomori stain for iron, and the Alizarin stain for calcium.

The effect of neodymium laser on intra-hepatic Vx2 carcinoma were studied by injecting a suspension of finely minced fresh tumor beneath the capsule of the liver at 2 to 6 sites in 24 rabbits. This model provided a

controlled amount of tumor for in vivo laser studies. Eight to 12 days after tumor implantation, the abdomen was opened and the small subserosal tumor nodules treated with laser energy. Following complete treatment of all visible tumor the abdomen was surgically closed. Some animals were followed for survival studies while others were sacrificed for histological sections.

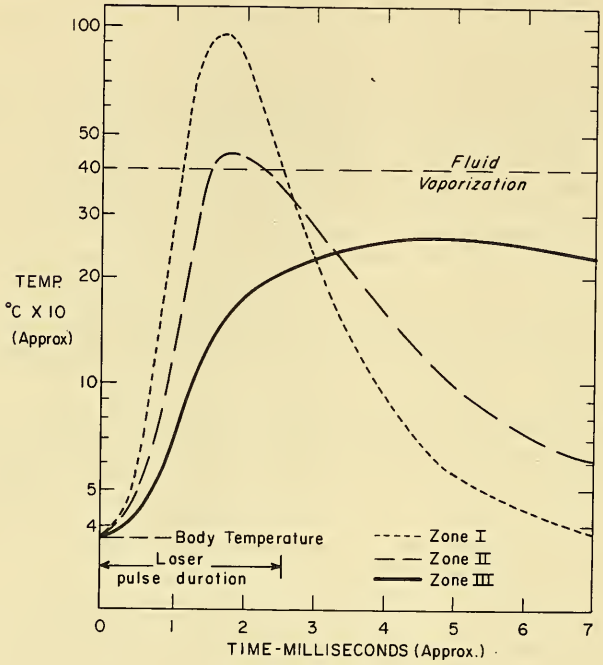
Major Findings:

The area of tissue treated with the laser showed different degrees of cell damage in both normal and tumor tissue. By six days post-laser, the fully developed laser lesion can be divided into three zones, each with a consistent and characteristic pattern of cellular damage. At the epicenter of the lesion (Zone I) the tissue had been vaporized and is absent. Surrounding the epicenter (Zone II) the tissue is twisted and distorted and many irregular shaped vacuolated spaces are present. Scattered throughout this zone are islands of cells and single cells which retain their normal staining characteristics and appear to be normal or only slightly damaged. Cells in mitosis are occasionally seen in this zone. Surrounding Zone II and extending to the periphery of the lesion is an area of complete cell death (Zone III). This zone is surrounded by a margin of fibroblastic activity.

Examination of the livers containing tumor, which has been treated with the laser, revealed small nodules of viable appearing proliferating tumor in the area of Zone II of the laser lesions, while small nodules proliferating tumor in the area of Zone II of the laser lesions, while small nodules in Zone III appeared to be necrotic. Of the six animals followed for survival studies after all of the tumor in the liver had been heavily treated with laser energy, none survived. At the time of death all animals had extensive intra-abdominal and intra-hepatic growing tumor.

These observations in normal and malignant tissues have permitted us to advance a thermodynamic analysis to explain the histological findings. The intense heat at the epicenter of the laser treated tissue results in complete destruction through vaporization of the tissue and this results in cavitation at the epicenter of the area treated. As the laser light passes into the area of tissue surrounding the epicenter, the temperature is no longer of a magnitude sufficient to cause tissue vaporization. Super-heating of the tissue fluid occurs causing gas formation and expansion. This results in vacuolization and distortion of the tissue in Zone II. Due to the more rapid dissipation and escape of gas, nearby cells are cooled. This cooling results in partial or complete preservation of cell structure in Zone II. As the laser energy passes into Zone III, the temperature produced in the tissue is less since neither vaporization nor super-heating of the tissue fluid occurs. The rate of energy loss through the tissue in this zone is slower than through the gas phase in Zone II, and the tissue remains heated for a longer period of time. This extended period of heating results in more intense cellular destruction and complete coagulation necrosis. A graphic description of

this process responsible for variable cell damage as a result of time and temperature relationships is represented in the attached figure.



Significance to Bio-medical Research and Program of the Institute:

These studies have demonstrated that with the highest amount of neodymium laser energy presently available even small tumor nodules on the surface of the liver cannot be completely destroyed.

This inability to cause complete destruction of all tissue treated is inherent in the biological properties of this type of laser tissue interaction. This finding has seriously curtailed the enthusiasm of our laboratory and that of others for the laser as an effective oncolytic tool. It indicates the need for further investigations prior to judicial clinical application.

Proposed Course of Project:

In recent years later tecnicological developments have not kept pace with the needs of the oncologist. Effective tissue destruction depends upon the wavelength of the laser, the energy density and the pulse length. These parameters cannot now be effectively manipulated to produce the desired result as they are not available or not reliable. For this reason, the majority of the laser investigations in this laboratory are being terminated.

We have modified a neodymium laser to produce a 2,000 joule pulse with a pulse length of 10 milliseconds. Studies of the biological properties of this laser system will be investigated in the near future.

Project Description:

Part II. Quantitation of some physical parameters associated with the laser tissue interaction.

Objectives:

It was the object of this study to investigate the temperature produced in tissue following an impulse of high energy neodymium laser, and to measure the pressures associated with the laser tissue interaction.

Methods Employed:

Temperature and pressure measurements were made on tumor bearing mice. The S91 melanoma and CDF_1 hybrid mice were used. Temperature measurements in tumor tissue at the focal point of the beam were attempted by insertion of a stainless steel sheath, No. 36 copper constantan thermocouple 6 mm. beneath the surface of a leg tumor, 1.5 cm. in diameter. A pulse of neodymium laser was then focused on the surface of the tumor and the temperature of the tissue as reflected in the temperature of the thermocouple recorded. Two types of pressure present in the laser tissue interaction were studied:

1. The force axial to and directly beneath the beam impacting upon the tumor site.
2. The airborne pressure wave associated with the "plume" of vaporized tissue resulting from the laser pulse.

In the first study, the animal was positioned on a wooden mounting board with the tumor site balanced directly above and in line with the Stratham Model UC-3 uni-directional transducer with a five pound load cell used as a sensor. The sensor signal was amplified through a Stratham Model UR-4 battery operated strain guage amplifier, coupled into one channel of a Tektronix type 1-A-1 dual amplifier in a Model 585 oscilloscope. Pressure measurements were recorded following focused impulses of from 250 to

950 joules to a 1 cm. S-91 melanoma tumor implanted on the dorsal flank of the mouse. The airborne "plume" pressure wave was measured by placing the animal in a sealed 10 x 10 cm. lucite box. Sensing and recording symptoms were identical to that just described, except that a Stratham type UGP-4-200 pressure diaphragm was substituted for the load cell. The animal was positioned to locate the impact site on the tumor surface at the mid-center of the box. The tumor was then treated with a focused 900 joule impact of neodymium laser.

Major Findings:

In the temperature measurements a 1,000 joule pulse was focused on the surface of the S-91 melanoma. The heat caused vaporization of parts of the tumor and also vaporized the stainless steel sheath and the thermo-couple which was placed 6 mm. beneath the surface of the tumor nodule. With the melting point of stainless steel at approximately 1500° C, it is concluded that even higher temperatures were generated by the pulse.

In the pressure measurements the force axial to and directly beneath the beam impacting the tumor site ranged from 300 to 800 lbs. per sq. in. The airborne pressure wave associated with the "plume" of vaporized tissue reached 400 lbs. per sq. in. when using a 900 joule focused impulse.

Significance to Bio-medical Research and Program of the Institute:

The parameters being measured in this portion of the project today have indicated that physical phenomena occurs at the time of laser tissue impact which may be detrimental to the tumor-host system since they may aid in dissemination of viable tumor tissue. Investigation of these physical parameters should lead to a better understanding of the laser tissue interaction. Through a satisfactory understanding and explanation of the physical factors involved, a method might be developed that would protect the host against any adverse effects which might be associated with the laser tissue interaction.

Proposed Course of Project:

Continued investigation of these parameters will be carried on by the Department of Bio-medical Engineering & Environmental Services. Through high speed motion picture photography, calculation of the speed of the particles contained in the "plume" of vaporized tissue is possible. A method of containing this "plume" and preventing its dissemination into the air is under testing at the present time.

Project Description:

Part III. The chronic effects of the laser tissue interaction.

Objectives:

To follow various experimental animals after laser treatment to determine what effects, if any, will develop in later years. Mice and monkeys are being used for these chronic studies at the present time. Both cutaneous and intra-abdominal laser impacts have been given these animals and they are being observed for late development and recurrent tumor or new tumor, as well as pre-malignant or malignant changes in the areas of laser treatment.

Major Findings:

No significant pre-malignant or malignant changes were observed in 200 mice which were followed for a period of 2 years after treatment of tumor on the dorsal flank with laser radiation. Fifteen of these mice developed evidence of neurological bladder dysfunction which eventually led to death but their were no other abnormalities noted. Eight monkeys were treated with multiple intra-abdominal laser impacts and are being followed for late changes in liver function studies. At eighteen months post-laser, four animals were lost, secondary to a tubercular epidemic in the colony and at this time there were no evidences of pre-malignant or malignant changes in the liver which had been previously treated heavily with laser radiation. The remaining 4 animals continued to be followed now 2 1/2 years post-laser, and show no evidence of change in liver function studies. In the future, biopsies of these lasered areas will be carried out.

Significance to Bio-medical Research and Program of the Institute:

In some areas of the country this form of photon energy is being used clinically for both benign and malignant lesions. It is important, therefore, to determine what, if any, late changes might be associated with this form of treatment.

Proposed Course of Project:

Animals presently on hand will continue to be followed for changes at the sight of laser energy application.

Project Description:

Part IV. Pathophysiologic studies of the effects of laser radiation upon normal and regenerating hepatic tissue in the rat.

Objectives:

1. To define by light microscopy and autoradiographic techniques, pathological and physiological effects of laser radiation distal to and at the sight of laser impact.

2. To study the healing phase and the effect of time upon the fate of the lesion.
3. To study by autoradiography the effect of laser radiation upon cell division distal to the sight of impact as a measure of subcellular or post-laser injury.

Methods Employed:

Male Sprague Dawly rates similar in age were divided into two study groups. Group 1 was comprised of animals which sustained a direct single laser exposure of 85 joules in 2.5 milliseconds to the normal liver through a standard laparotomy wound. Group 2 consisted of animals which were treated in a similar fashion with laser at varying time intervals following partial hepatectomy. Autopsy in both groups was carried out at intervals varying from 1 to 14 days following intra-peritoneal injections of tridiated thymidine for the purpose of labelling cells in mitotic division. Control animals were matched to each study animal and were subjected to all study conditions except exposure to laser radiation. Following fixation and sectioning, specimens were subjected to hematoxlyn and eosin stains and paired sections treated with standard autoradiographic techniques.

Major Findings:

All of the autoradiographic sections have not yet been developed. Preliminary review of the histological section thus far reveals the same evidence of variable degrees of cell damage that was evident with a pulse of 1,000 joules in the rabbit liver. No thymidine granules have been noted as yet in the capacity of the cells under the laser treated area to divide when compared with untreated liver tissue. Further conclusions must await complete analysis of the data.

Significance to Bio-medical Research and Program of the Institute:

Besides heat and pressure there is associated with the laser tissue interaction acoustic waves and ultrasonic waves. All of these properties have the compacity to destroy or seriously interfere with the function of cells surrounding the area of laser treatment. This experiment will answer the question, "Are the cells surrounding the point of laser treatment damaged and to what extent are they damaged?"

Proposed Course of Project:

Further extension of this work awaits complete analysis of the data.

Project Description:

Part V. A study of the ultrastructure of cells at a distance from the laser tissue interaction.

Objectives:

The lesion produced by laser energy in liver tissue is well localized and sharply demarcated from the surrounding normal tissue. With light microscopy there is no structural alteration in the tissue outside of the laser impact site. However, electron microscopic study of liver sections in primates following minimal shock, have demonstrated marked alterations in structures, not apparent with light microscopy. The objective of this pilot study is to investigate two laser lesions (low energy and high energy). The areas studied with the electron microscope will include the area of liver directly under the lesion and an area of the liver remote from the impact site.

Methods Employed:

New Zealand white rabbits were employed in the study. Two laser lesions were produced in the liver, one consisting of 900 joules and the second consisting of 300 joules energy per pulse. At 7 days following laser treatment the animals were sacrificed and histological sections made of each lesion. A portion of the normal appearing liver tissue directly under the point of impact and not apparently affected by the laser treatment was submitted for electron microscopic study. In addition a small portion of liver tissue remote from the site of the laser impact was similarly treated. Electron photographs were made of the cells, the nuclei, and the blood vessels in the tissue underlying the laser impact site.

Major Findings:

Processing of the tissue and photographs has only recently been completed and complete analysis of the data is not yet available.

Significance to Bio-medical Research and Program of the Institute:

The laser has been proposed as a tool for the treatment of cancer. However, such a small volume of tissue is involved with the laser tissue interaction that use of the laser for this purpose does not seem reasonable. If more distant anatomical and/or physiological effects are associated with the interaction of the laser with tissue, than potentiation of these effects by chemical or other physical means could result in a widespread area of cell damage. This study will identify any damage not apparent when using light microscopy.

Proposed Course of Project:

Further extension of this work must await final analysis of this pilot study.

Project Description:

Part VI. The clinical application of laser energy.

Objectives:

To evaluate the clinical use of laser energy to destroy multiple cutaneous melanoma implants with and without the use of adjuvant therapy.

Methods Employed:

A high-energy, neodymium pulsed laser unit has been constructed by the U.S. Army Missile Command in Huntsville, Alabama, for the purpose of carrying out this project. An operating room on the tenth floor of the Clinical Center has been remodeled so as to permit the safe use of laser energy in a clinical situation.

Major Findings:

Following installation of the laser in the operating suite, repeated modifications and testing indicated that the unit as constructed was not technically satisfactory for use on patients. Extensive remodeling and re-design of the laser was necessary before an adequate pattern distribution of the laser light resulted. During these setbacks our experimental work continued in the laboratory. Results from these latter studies have indicated that the cellular damage produced by the laser tissue interaction is not sufficiently complete to warrant its use clinically. Moreover, the adverse effects associated with dissemination of viable tumor cells prevent us from continuing this line of investigation. The laser, since installation has been modified so as to double the amount of energy output and increase the pulse length from 2 milliseconds to 10 milliseconds. It is quite possible that with this change in the physical parameters of the laser system that the histological pattern of destruction may be quite different. This new laser system has not yet been tested on living tissue.

Significance to Bio-medical Research and Program of the Institute:

Laser is a unique form of high intensity photon energy which has been shown to be effective in destroying some small animal tumors. Its exact usefulness in the destruction of human tumor remains to be evaluated in a well designed and controlled study.

Proposed Course of Project:

Further extension of this clinical project awaits testing of this recently modified laser unit on living animal tissue. If the pattern of cellular damage is uniform and complete then clinical testing may be indicated.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Hoye, R.C., Weiss, G.H., Riggle, G.C., Ketcham, A.S. and Gart, J.: Potentiation of laser oncolysis with pretreatment x-irradiation. Radiat. Res. 32: 112-116, Sept. 1967.

Ketcham, A.S.: The laser--its role in cancer. Ca 297-300, Nov.-Dec. 1967.

Ketcham,A.S., Hoye, R.C. and Riggle, G.C.: A surgeon's appraisal of the laser. Surg. Clin. N. Amer. 47: 1249-1263, Oct. 1967.

Mullins, F., Hoye, R.C., Ketcham, A.S., Kelly, M., O'Gara, R., McKnight, W.B. and Jennings, B.: Studies in the laser destruction of chemically induced primate hepatomas. The Amer. Surg. 33: 298-303, Apr. 1967.

Riggle, G.C. and Hoye, R.C.: Effects of laser energy on living tissue. IEEE Internatl. Conv. Record 9: 95-96, 1967.

Riggle, G.C., Hoye, R.C., Ketcham, A.S. and Hawkins, R.W.: Development of a high energy neodymium laser system for surgical use. Med. Biol. Engin. 164-165, 1967.

Serial No. NCI-3812

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Viral Oncogenesis

Previous Serial Number: Same

Principal Investigator: Dr. Willis E. Brown, Jr.

Other Investigators: Dr. A. S. Rabson, Dr. Ruth Kirschstein, Dr. Ira Black, Dr. S. K. Orme, and Dr. S. A. Wells

Cooperating Units: Surgery Branch, NCI; Department of Pathologic Anatomy, NCI; Laboratory of Pathology, DBS; Laboratory of Clinical Science, NIMH.

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1/2
Professional:	5
Other:	0

Project Description:

Part I. Viral transformation of adult primate pineal tissue

Project Description:

Part I. Viral transformation of adult primate pineal tissue

Objectives:

To develop techniques of pinealectomy in adult Rhesus monkeys and to establish explant cultures of pineal tissue were the primary objectives of this project. Secondarily, we wished to attempt oncogenic transformation of the tissue in culture, to investigate transplantation of this tissue and the functional activity of the transformed cells.

Methods:

Three to four Kg Rhesus and Virvet monkeys underwent post-mortem pinealectomy and standard explant tissue cultures were established. Similar explant cultures were established with pineal tissue derived from monkeys who underwent and recovered from pinealectomy. Transformation was attempted with unmodified and irradiated SV40 virus in vitro.

Major Findings:

The technique of pinealectomy has been refined and the final 4 of a series of 8 monkeys have recovered from the procedure without serious deficit.

Explant cultures of pineal tissue have been established with regular success more recently. However, the first series of attempts to achieve in vitro transformation of explanted tissue with unmodified SV40 virus was not successful, as lysis of the cultures followed infection with the virus. A second series of cultures was therefore exposed to irradiated SV40 virus and the culture observed for transformation. The Rhesus pineals early exhibited evidence of in vitro transformation, but were subsequently lost, possibly due to the effects of the virus. In contrast, Virvet tissue has exhibited in vitro characteristics of transformation and has been carried through 8 or more subcultures.

Transplantation of the transformed Virvet cells had been delayed because of the recent difficulties in obtaining Virvets. In two instances, intracerebral innoculation of cells into neonatal monkeys produced no clear evidence for growth, as distinguished from persistence, of the cells.

Assay of cells from in vitro status by Dr. Ira Black for the enzyme specific to the pineal, Hydroxy-indole-ortho-methyl transferase, demonstrated barely detectable levels, a result perhaps compatible with the in vitro status of the cells.

Significance to Bio-medical Research and Program of the Institute:

Although transformation in vitro of rodent neonatal pineal tissue has been achieved, we hope to establish the possibility of such transformation in adult primate pineal tissue. The demonstration of HIOMT activity in transformed, transplanted cells of primate origin would add to the body of material on the functional status of cells transformed by oncogenic virus and might produce a model of a tumor growing in a primate with an enzyme tag which is specific for the tumor.

Proposed Course of the Project:

Attempts to transplant in vitro transformed cells to adult monkeys are in progress. If successful growth is achieved, assay of the transformed, transplanted tissues for HIOMT will be undertaken.

Serial No. NCI-3813

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Surgical Endocrinology

Previous Serial Number: Same

Principal Investigator: Dr. John R. Marshall

Other Investigators: Drs. Alfred S. Ketcham, Arnold Jacobson, Griff T. Ross, Charles B. Cargille and Ralph Powell

Cooperating Units: Endocrinology Branch, NCI; Pathologic Anatomy Branch, NCI; Reproductive Biology Branch, NICHD

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Part I. Pharmacology of Ovulation Induction with Gonadotropins.

Part II. Plasma and Urinary HCG levels in Early Pregnancy.

Part III. Gonadotropin Control: Clomiphene

Project Description:

Part I. Pharmacology of ovulation induction with gonadotropins.

Objectives:

1. To clarify the significant variables in ovulation induction with human menopausal gonadotropins.
2. To develop a treatment technic which is a safe, effective and widely applicable method.

Methods Employed:

Using a standard threshold dose which was determined by means of individual

patient dose response curves and which gives a 70% ovulatory response rate variables controlling response are studied in anovulatory and infertile women.

Major Findings:

Estrogen response at subovulatory doses is dose related. Ovulatory response rate at ovulatory doses is dose related. Ovarian enlargement rate is positively dose related. Oligo-ovulatory patients have lower thresholds than do anovulatory patients. Effectiveness of varying dosage schedules is determined by patient threshold. Patients requiring less than 1900 i.u. FSH/cycle respond to a single injection of the entire dose; patients requiring larger gonadotropin dose need divided doses. The FSH and LH content of the material are both of importance in determining ovulatory rate, however, FSH content seems to be more critical. Low LH materials are less effective in inducing ovulation than are materials with greater amounts of LH. Pre-treatment urinary gonadotropin levels are not determinants of responsiveness unless very high levels are found. Urinary steroid studies likewise are not helpful in selecting dose. Plasma LH is of some help in predicting gonadotropin dose. Plasma FSH levels are of little use unless markedly elevated.

Based on the responses noted so far a treatment schedule has been developed which is safe, effective and widely applicable.

Significance to Bio-medical Research and Program of the Institute:

Human menopausal gonadotropin promises to be a useful agent in the treatment of anovulation in women desiring conception. The present problems associated with this drug are such as to preclude its widespread and unregulated use. It is our feeling that these unwanted side effects are largely dose related and could be eliminated by more appropriate selection of dosage. In this regard, the information derived from this study will provide a basis for valid selection of dosage for the individual patient. In this way, ovulation will be induced with a lower and more acceptable incidence of unwanted side effects.

Proposed Course of Project:

Project is continuing with attention to additional variables and to more basic physiology of response.

Project Description:

Part II. Plasma and urinary HCG levels in early pregnancy.

Objectives:

To measure plasma HCG levels in early pregnancy.

Methods Employed:

Plasma HCG assay of Odell, Ross and Rayford of plasma from patients pregnant following ovulation induction with HMG/HCG.

Major Findings:

Plasma HCG concentrations are elevated over control values by conception day 11 and thereafter rises exponentially until at least day 21. Geometric consideration of the developing ova would suggest that the trophoblast mass increases as does the diameter to the second power. When the maximum diameter of the trophoblast squared (early human ova collected by Hertig and Rock) is compared to the rate of increase of HCG concentration no significant difference is seen. This suggests that plasma HCG concentrations mirror trophoblast mass.

Significance to Bio-medical Research and Program of the Institute:

Chemotherapy of gestational trophoblast neoplasia has been based upon the assumption that gonadotropin excretion adequately reflects residual tumor. The present study strongly substanciates this assumption.

Proposed Course of Project:

Publication of data.

Project Description:

Part III. Gonadotropin control: Clomiphene

Objectives:

To determine the effects of Clomiphene on gonadotropin release.

Methods Employed:

Patients under study for anovulatory infertility were treated with clomiphene and plasma gonadotropin concentrations measured and correlated with various clinical parameters.

Major Findings:

Clomiphene causes a rise in plasma FSH concentration beginning 1 day following initiation of the therapy. Plasma LH rises more slowly and reaches an initial peak about day 6. Both hormones then fall to nearly normal levels only to peak just prior to initiation of the luteal phase.

Significance to Bio-medical Research and Program of the Institute:

Clomiphene is a widely used agent for ovulation induction. Its method of action has not been well understood. It is, additionally, now shown to be a useful test of pituitary function.

Proposed Course of Project:

Publication. Additional patients will be tested using Clomiphene as a pituitary stimulant.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL;

Bardin, C.W., Lipsett, M.B., Edgecomb, J.H. and Marshall, J.R.: Studies of testosterone metabolism in a patient with masculinzation due to stromal hyperthecosis. N. Eng. J. Med. 277: 399, 1967.

Serial No. NCI-3814

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30,1968

Project Description: Physiology of the Female Reproductive Tract

Previous Serial Number: Same

Principal Investigator: Drs. Willis E. Brown, Jr., John L. Lewis, Jr.

Other Investigators: Drs. Robert Johnson, Robert Davis and Griff T. Ross

Cooperating Units: Endocrinology Branch, NCI

Man Years: (July 1, 1967 through June 30, 1968)

Total:	1
Professional:	1/2
Other:	1/2

Project Description:

Part I. Investigation of the Growth of Human Choriocarcinoma in Sub-Human Primates.

Project Description:

Part I. Investigation of the Growth of Human choriocarcinoma in sub-human primates.

Objectives:

To develop and characterize a model of human choriocarcinoma growing in the brain of a sub-human primate.

Methods Employed:

Woods strain of human choriocarcinoma, which has been propogated in Syrian hamster cheek pouches for over 300 generations without change in morphology, histopathology or endocrine function, was transplanted uner sterile conditions into the cerebral cortex of 10 adult Rhesus monkeys and innoculated intracerebrally into 4 neonatal cynomologous monkeys. The histopathology and viability of the tumor were followed with routine microscopic sections and by simultaneous transplantation of matched pieces of tumor into hamsters.

Modifications of routine carotid angiographic and Technetium 99^{m} brain scanning techniques were employed in the adult monkeys. Bioassay and radio-immunoassay were employed to estimate levels of human chorionic gonadotropin in serum and cerebrospinal fluid selected monkeys.

Major Findings:

The transplantation of human choriocarcinoma to the brain of monkeys has been achieved in adult and newborn animals.

In 10 adult Rhesus monkeys studied consecutively, transplanted human choriocarcinoma grew rapidly, was manifest by carotid angiography in 10 of 10 instances and by brain scan in 5 of 5 instances. The monkeys succumbed to the effects of rapid tumor growth or were killed in a moribund state in 9 of 10 instances. The range of death time was 16 to 23 days with an average death time of 20 days after transplantation. In 10 of 10 monkeys viable tumor was recovered from the monkey brain when the monkey died. This viability was demonstrated by growth of the tumor in hamster cheek pouches. Functional activity of the tumor, i.e. production of human chorionic gonadotropin, has been demonstrated in selected animals by bioassay and by radio-immunoassay. Preliminary radio-immunoassay data suggest that there is a rising titer of human chorionic gonadotropin at the time of monkey death. Although Burro anti-Rhesus monkey antilymphocytic serum was administered to 5 of the 10 adult monkeys, no differences in tumor growth were detected with regard to death time, gross or microscopic appearance of the tumor.

In a series of 4 neonatal monkeys, growth of the tumor was achieved in 3 of 4 instances with viable tumor being demonstrated in vivo in 2 of 4 instances. Because of the difficulties in working with neonatal monkeys and because of the successful growth of this tumor in adult monkeys, we have not further studied transplantation to neonatal monkeys.

Significance to Bio-medical Research and Program of the Institute:

The growth of human choriocarcinoma in monkeys has not been previously achieved, despite the use of thymectomy, immunosuppressive, and hormonal drug therapy. This model presents, then, for the first time a sub-human primate model for the study of some general aspects of human choriocarcinoma in a primate and specifically the growth of human choriocarcinoma in a primate brain. Problems of diagnosis and treatment of intracranial foci of choriocarcinoma may be approached through this model.

Proposed Course of Project:

At present under active study are the following aspects of this tumor system: definition of suitable controls for radio-immunoassay of human chorionic gonadotropin in monkey plasma; assessment of plasma and cerebrospinal fluid HCG levels in monkeys bearing tumors; studies of growth of tumor in sites other than the cerebral cortex and in the brains of

other laboratory animals; the degree of immunologic privilege afforded by the brain and the fate of the transplanted tumor.

Publications:

ARTICLES PUBLISHED IN A PERIODICAL:

Davis, R.C. and Lewis, J.L.: Effect of thymectomy on an anti-lymphocytic serum treated human tumor xenograft. Surg. Forum 18: 229-231, 1967.

Lewis, J.L.: Chemotherapy of metastatic gestational trophoblastic neoplasms. Clin. Obstet. Gynec. 10: 330-341, June 1967.

ARTICLES IN PRESS:

Brown, W.E., Jr.: Transplantation of human choriocarcinoma to the brain of Rhesus monkeys. (Proceedings of the Fourth Rochester Trophoblast Conference)

Lewis, J.L., Jr., Brown, W.E., Jr., Hertz, R., Davis, R.C., and Johnson, R.H., Jr.: Heterotransplantation of human choriocarcinoma in monkeys. (Can. Res.)

Serial No. NCI-3815

1. Surgery Branch
2.
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1967 through June 30, 1968

Project Title: Tissue and Organ Transplantation

Previous Serial Number: None

Principal Investigator: Dr. Donald L. Morton

Other Investigators: Dr. Alfred S. Ketcham, Dr. Yosef Pilch, Dr. William Hammond, Dr. E.C. Holmes, Dr. Barry Kahan, Dr. Ralph Reisfeld, Dr. Kenneth Ramming and Dr. Frank Sparks

Cooperating Unit: Laboratory of Immunology, NIAID

Man Years: (July 1, 1967 through June 30, 1968)

Total:	2
Professional:	2
Other:	0

Project Description:

Part I. Studies in lung transplantation

Part II. Isolation of guinea pig transplantation antigen from methylcholanthrene-induced sarcomas

Part III. Isolation of tumor specific antigens from methy-cholanthrene-induced sarcomas

Part IV. In vivo immunosuppressive effects of Phytohemagglutinin

Part V. Detection of humoral antibodies to histocompatibility factors

Project Description:

Part I. Studies in lung transplantation

Objectives:

1. To determine the technical feasibility of lung transplantation.

2. To study pulmonary physiology and evaluate the functional capacity

of unilateral and bilaterally transplanted lungs.

3. To develop immunosuppressive techniques which will permit prolonged survival of homotransplanted lungs

Methods Employed:

1. The technical feasibility of lung transplantation will be evaluated in monkeys, dogs, and baboon. All initial studies will be unilateral re-implantation of the entire lung or the left lower lobe. When this has been successfully accomplished, we plan to do a re-implantation of the contralateral right lung or the right lower lobe.

2. The functional capacity of the transplanted lung will be evaluated by blood gas studies, serial lung biopsies, pulmonary function testing, and exercise tolerance in animalswho have undergone unilateral or bilateral re-implantation of the lower lobes or the entire lung.

3. Immuran, which has been so successful as an immunosuppressive agent for the prolongation of kidney homografts in dogs, has not been equally successful when used in lung transplantation. Therefore, we plan to evaluate the effectiveness of two other possible immunosuppressive techniques. Antilymphocyte serum has been used successfully by Drs. John Lewis and Robert Davis to prolong survival of skin allografts in monkeys and tumor heterografts in hamsters. We plan to evaluate the effectiveness of antilymphocyte serum in the prolongation of lung homografts. However, perhaps the most promising immunosuppressive technique from a theoretical standpoint would be the development of a means for establishing a permanent thoracic duct intestinal fistula. We are currently working on the technical problems associated with creating a permanent thoracic duct fistula.

Major Findings:

Many of the technical problems relating to surgical technique, and types of anesthesia have been solved. We have performed left lower lobe re-implantations in eight dogs. Five of these are alive and well ten months following auto-transplantation of the left lower lobe. Autotransplantation of the left lower love has been attempted in three baboons with one successful re-implantation. This animal was sacrificed six months following auto-transplantation at which time it was found to have a normally functioning left lower lobe. Lung transplantation has been attempted in 12 monkeys with 2 successful. These two monkeys are now alive one year following re-implantation of the left lower lobe.

Significance to Bio-medical Research and Program of the Institute:

There is a great potential need for lung transplantation among patients with chronic pulmonary emphesema, pulmonary fibrosis, children with cystic fibrosis, and patients with severe pulmonary disease who develop carcinoma of the lung and need resectional surgery. Therefore, the development of a successful lung transplant program would have almost unlimited clinical application.

Proposed Course of Project:

We plan to continue this project in the manner outlined above in the hope that the laboratory program will be sufficiently successful that it will warrant eventual clinical application.

Project Description:

Part II. Isolation of guinea pig transplantation antigen from methylcholanthrene-induced sarcomas.

Objectives:

1. To determine if a water soluble transplantation antigen can be isolated from a guinea pig sarcoma.

2. To purify this antigen.

3. To compare the amino acid composition of this antigen to that of transplantation antigens isolated from normal guinea pig tissues.

Methods Employed:

A cell suspension of the tissue (malignant or normal) was exposed to sonic oscillation. The cell membranes were removed by centrifugation at 130,000 xg. This cell suspension was then further purified by passage through sephadex G-200 followed by discontinuous electrophoresis on polyacrylamide gels. The active fractions are being analyzed for amino acid composition.

Major Findings:

Specific delayed cutaneous hypersensitivity reactions were elicited in allogenic hosts sensitized with tumor by an electrophorectic component with the same Rf value as that isolated from spleen, lung and liver tissues. Preliminary data suggest that amino acid composition of the tumor and normal tissue fractions are quite similar.

Project Description:

Part III. Isolation of tumor-specific antigens from methylcholanthrene-induced sarcomas

Objectives:

An attempt is being made to isolate a water soluable tumor-specific antigen from MCA induced guinea pig sarcomas and an attempt will be made to identify humoral tumor-specific antibodies in the sera of immunized animals.

Methods Employed:

A single cell suspension of tumor cells are disrupted by sonic oscillation. The cell membranes are removed by dentrifugation at 130,000 xg. The supernatant is then fractionated on a sephadex G-200 column. Various fractions are then injected intradermally into animals who have been previously sensitized with whole tumor in an attempt to elicit a specific delayed cutaneous response.

The serum from these immunized guinea pigs will than be analyzed by the anti-globulin consumption test for the presence of tumor-specific antibodies.

Major Findings:

The findings to date indicate that a tumor specific component may be present in one of the sephadex fractions as determined by delayed cutaneous hypersensitivity. However, the number of animals are small and further studies using larger numbers of animals are in progress. There is no data at present on the presence or absence of tumor-specific antibodies in the sera of these animals.

Project Description:

Part IV. In vivo immunosuppressive effects of Phytohemagglutinin

Objectives:

To study and compare the immunosuppressive effects of Phytohemagglutinin with agents now being used in clinical organ transplantation.

Methods Employed:

Phytohemagglutinin (PHA) is administered in varying doses intravenously to adult mongrel dogs on a daily regimen and the following observations are made: 1) the effect on skin allograft survival; 2) the effect on antibody production to protein antigens and heterologous red blood cells when the agents are given (a) prior to PHA (primary immunization) and again after PHA is begun, (b) immediately after initiation of PHA, and (c) several weeks

after PHA is begun. The RBC agglutination test is used to quantitate antibody production in these tests; 3) These studies are repeated in animals receiving a low and clinically ineffective dose of Azothioprine alone and in combination with the PHA regimen used in 1 and 2. The morphological alterations in the lymphoid tissue and bone marrow, and the effect on the lymphocyte levels in the peripheral blood are also studied, as well as levels of blood immunoglobulins.

Major Findings:

PHA administered in maximum non-toxic doses results in slight prolongation of skin allograft survival that is of no value per se clinically. When this dose regimen is combined with a low dose regimen of Azothioprine that has a similar mild effect on graft survival, permanent graft survivals in the dogs studied thus far have resulted, with grafts in the first dogs so studied now surviving 50 to 90 days. Leukopenia occurs with this combined regimen, however, no adverse constitutional symptoms of any kind have been observed with PHA. This is contrasted with the toxic facts observed with Azothioprine alone in sufficient doses to produce similar leukopenia. One explanation for the phenomenon is that it is due to the stimulation of antibody production by PHA against bacterial antigens to which the animal has been exposed prior to PHA therapy. The studies on antibody production are designed to give evidence for the validity of this hypothesis, however, the results of this portion of the experiment is not available at this time.

Significance to Bio-medical Research and Program of the Institute:

Successful surgical therapy of certain malignancies in organs such as lung, liver and abdominal viscera may require transplantation of these organs. Since the donor organs implanted would therefore of necessity be obtained from cadavers (unrelated donors), potent yet well tolerated immunosuppression would be indispensible for survival of the transplant. Durgs having these characteristics are not at present available. Although lethal or near lethal doses of presently-used immunosuppressants frequently does not prevent injection of grafts from unrelated donors, preliminary observations on the effects of PHA suggests that it does have some of these desirable characteristics.

Proposed Course of Project:

The adjunctive effects of PHA with anti-lymphocyte serum and steroids will be studied using the test system utilized to study its adjunctive effect on Azothioprine immunosuppression. Later evaluation of the adjunctive effect of PHA in combination with all these drugs will be evaluated in lower primates and humans. We anticipate that eventually PHA will be used in clinical transplantations, and that it may be the best tolerated and most potent immunosuppresant available.

Project Description:

Part V. Detection of humoral antibodies to histocompatibility factors

Objectives:

To study different parameters of and evaluate the usefulness of the indirect isotope-labeled antibody technique for studies in tissue transplantation.

Methods Employed:

Antisera are raised in inbred mice against allografts of normal tissue from other inbred strains differing from the antisera producers at strong H-2 histocompatibility loci. Single cell suspension, labeled with radiochromium from tumors, spleens, or lymph nodes of the immunizing strain are incubated with the antiserum at room temperature for ½ hour and are then washed and centrifuged 8 times. The target cell-antibody complex is then incubated with ^{125}I labeled antibody against mouse immunoglobulin and then washed and centrifuged 8 more times. The amount of ^{51}Cr and ^{125}I in the mixture is determined by differential isotope counting. The amount of ^{125}I labeled antibody per standard number of ^{51}Cr labeled target cells is calculated. Comparison of these values with those obtained by carrying out a similar procedure using normal mouse serum instead of antiserum gives an immune/normal ratio, which is thus indicative of the amount of antibody present in the antiserum.

Major Findings:

Much exploratory effort has been expended in determining the optimal concentrations of target cells, antiserum, and indicator globulin, and the optimal conditions for their interactions. In antisera prepared by hypersensitization of the animals, consistent and significant elevations of antibody levels have been obtained. Preliminary results suggest that detectable antibody elevations are present during skin allograft rejection in previously unsensitized mice.

Significance to Bio-medical Research and Program of the Institute:

Thus far the only satisfactory method for demonstrating humor antibody to histocompatibility factors has been the use of cytotoxicity tests. Since this is of value only when cytotoxic antibody is made, and since in many allograft systems cytotoxic antibodies are not present, the usefulness of a test which measures total immunoreactive antibody will be considerable.

Proposed Course of Project:

The method will be used in studies designed to ascertain the relative roles of humoral antibody and cellular immunity in the allograft rejection process. Also, it is likely that tumor-specific antibody will be demonstrable by this

method and investigations will be carried out to study similar problems in tumor immunology.

Publications:

ARTICLE PUBLISHED IN A BOOK:

Perry, S., Whang, J. and Irvin, G.L.: Studies of lymphocyte kinetics in man. The Lymphocyte in Immunology and Haemopoesis Chapter 12: 99-107, 1967 ed. Yoffey

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